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Erratum.

Vol. 13 pag. 361 line 12 from above, for "20 per cent" read "2.0 per cent".

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The Contents of Phytic Acid P in Norwegian Flour and Bread Types II.

Factors Influencing Phytic Acid Decomposition during Breadmaking and the Contents of Phytic Acid P in Ordinary Norwegian Bread.

By

ARNE SCHULERUD.

Received 12 March 1947.

In a previous paper the author (1944) has determined the amounts of phytic acid P in various flours, and the rate of their enzymatic decomposition in water suspensions. Among the cereals, rye was found to have the greatest phytase activity, followed by wheat and barley, while oats had none at all. In the meantime, several Danish examinations partly have confirmed the author's findings, such as the greater phytase activity of rye, and partly thrown light over new sides of the phytic acid problem, both over its technological and its clinical side. MÖLLGAARD et al. have demonstrated the effect of various organic acids as "activators".

The influence of phytic acid in the food on the absorption of phosphorus, calcium and iron, although still under discussion, makes an exact knowledge of the contents of phytic acid compounds in the more important bread types desirable. This is a task which must be solved for every country separately, since flour types, baking methods and other factors will have a great influence upon the rate of enzymatic decomposition in the baking process. In the present paper, the author will give a survey of the usual Norwegian bread types in this respect, in connection with some examinations concerning more general sides of the phytic acid problem.

I. Conditions for the Decomposition of Phytic Acid P in Doughs.

a) *Quality of the flour.* First comes the influence of the kind of the cereal, rye having the greatest phytase activity. Barley and oats usually do not appear in fermented bread, but during the war these cereals made quite a high percentage of Norwegian baking flour. The effect of these additions together with CaCO_3 , which was also added, will be treated in another publication.

Then follows the degree of flour extraction. In the seed, phytic acid is located in the outer layers and the bran, and consequently the amounts will increase with increasing extraction. In straight flours, there is a close correlation between ash contents and the quantity of phytic acid P, which can be expressed by the equation

$$\% \text{ phytic acid P} = \% \text{ ash} \cdot 0.16 - 0.036$$

This equation, based upon the examination of 34 different flours, has a correlation coefficient of $+0.935$, and is valid for straight flours from wheat, rye and barley, and permits an approximate calculation of the percentage of phytic acid P in such flours, when the ash contents is known. Oat products and bran do not follow this equation.

b) *Water contents of the doughs.* In a dough, most of the water is bound colloiddally to the swelling proteins. Since the decomposition of the phytic compounds is dependent upon diffusion and solution, the process will be influenced by the amounts of free water present, and a slack dough give better conditions than a stiff one. From the author's experiments, an illustration to this is given in Figure 1.

The baker wants a definite consistency of his doughs, and will choose the proportion flour: water to give the proper stiffness. This ratio will vary with the flour quality and with additions such as salt, sugar and acids, and also from one bakery to another, giving great variations in the consideration of this factor.

c) *Dough temperature.* Several investigations have been made on the thermoresistance of the phytase. By low temperatures its activity is small, but an increase in temperature from 20 to 30° C seems to accelerate the decomposition process with some thirty percent. Normally, dough temperatures do not exceed the interval 25—35° C. However, since the enzyme has its optimum

at 55° C and hardly is inactivated till above 70°, its activity will proceed during the first time the loaves are in the oven, and may play an important part. How far this oven effect will go, depends upon how fast the heat penetrates into the loaves, that is the size of the loaves and oven temperature. At elevated temperatures the phytase pH optimum is moved into a more acid direction.

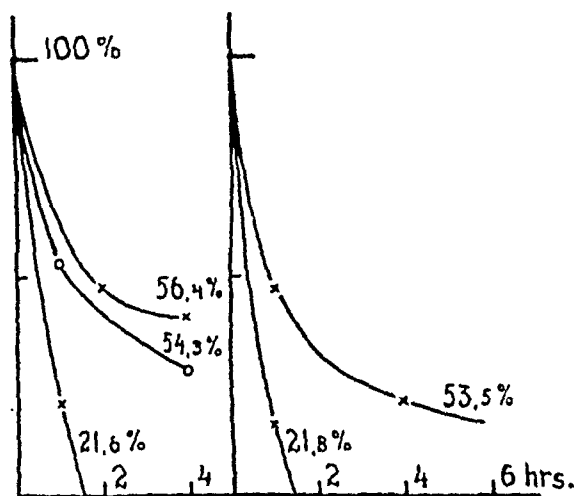


Figure 1. Rate of phytic acid decomposition in doughs with different dry matter contents. Left: Wartime flour of whole rye with 15 % barley and oats plus an addition of 0.4 % CaCO_3 , right pure whole rye without calcium addition.

Big loaves made from sour dough and slowly penetrated by the heat will offer most favourable conditions for the decomposition of eventually remaining phytic acid P, and better, the sourer the dough.

d) *The dough scheme.* It is evident that phytic acid decomposition will go farther the longer a dough is allowed to rest. In practical baking this dough rest is, and must be, different for different bread types. Wheat generally has a short scheme, rye a long one. In sour dough baking the process goes through several steps, new amounts of flour and water being added each time. All phytic acid may be split before every new addition, and sour dough therefore is the best scheme in this respect, but it is limited to rye and even to such bread where the sour taste is not disapproved of. In Norway, where sour bread is not wanted, the sour dough is substituted by a sponge dough, giving very little lactic acid fermentation overnight. In the sponge, full decomposition can be obtained, but the development in the final dough depends on further conditions. Whole meal wheat bread may also be made

with a sponge, but white bread usually by a "direct" dough, resting a couple of hours only.

e) *Acidity of the dough.* At room temperature, phytase activity has its optimum at pH 5.2—5.4. In wheat doughs, pH will be about 5.8—6.2, and the conditions thus not very favourable. They are better in rye doughs, even if they are not made with sour dough. If pH of the doughs can be adjusted to about 5.3 with small amounts of a suitable acid, such as lactic acid, decomposition rate can be favourably influenced, and this can be done in some cases without harm to the baking quality or the taste of the loaf. At elevated temperatures, in the oven, the pH optimum moves into the sour direction. However, the process is not influenced by pH only, but also by the kind of acid anion, and since the picture is contaminated in the presence of calcium salts, it is necessary to treat these questions separately.

II. Effect of Acid Anions on Phytic Acid Decomposition Alone and in Presence of Calcium.

MÖLLGAARD et al. have reported that organic oxy acids act as activators on phytic acid decomposition, with tartaric and citric acid as the most effective in doughs containing 0.5 % Ca. HOFF-JÖRGENSEN and PORSDAHL, however, find lactic acid better in their experiments with Na-phytate solutions. In MÖLLGAARD's publication a great number of organic acids has been studied, but a full evaluation of the results is not possible, since in the same series of experiments different flours have been used, with different amounts of total P, phytic acid P, and very probably different phytase activity. Besides, the experimental doughs have not been adjusted to the same pH, which is very important. From MÖLLGAARD's tables, the author has extracted the data which are comparable, being derived from the same flour at the same pH, and thus found the following order of activity of the acids:

1. Tartaric acid
2. Citric acid
3. Malic acid
4. Lactic acid
5. Pyruvic acid
6. Gluconic acid

With bran extracts in Na-phytate solutions, HOFF-JÖRGENSEN and PORSDAHL find this order:

1. Lactic acid
2. Citric acid
3. Tartaric acid
4. Acetic acid.

For the activating effect, it is agreed that the active principle is the OH-group of the acids. For instance, acetic acid has very little effect, while the corresponding OH-substituted acid, glycolic acid, in MÖLLGAARD's studies seemed very active. In presence of calcium ions, phytic acid will precipitate an insoluble Ca-pentaphytate, which will greatly retard the work of the phytase. Now, bi- or tricarbonic oxy acids have the power of bringing this salt into complex solution and thus restoring to some extent the conditions for enzymatic splitting. Since monocarbonic acids have not this power, they will be inferior in presence of calcium, and thus tartaric and citric acid were found best by MÖLLGAARD, while lactic acid was better in the Ca-free Na-phytate medium of HOFF-JÖRGENSEN—PORSDAHL.

The effect of acids on phytase, then, seems to consist of three factors:

1. Hydrogen ion concentration
2. Chemical constitution and dissociation
3. Power of forming complex, soluble salts.

Factor 3 is of importance in presence of calcium only. Further, calcium must be present in soluble state. If it is added as CaCO_3 , which was usual during the war, the dough must be sour enough to bring it into solution. In the author's experiments, no effect was found when pH of the dough was 6 or above. However, Danish rye bread is sour, and so was Norwegian bread during the war, and here the retarding effect on phytic acid decomposition must be expected. As for the hydrogen ion concentration any enzyme has its optimum at a definite pH, and in this respect is independent on the kind of acid used, except that the stronger the acid, the smaller the quantity necessary to give this pH. Both MÖLLGAARD and HOFF-JÖRGENSEN—PORSDAHL operate with definite quantities of organic acids and adjust pH by addition of HCl or NaOH, but we are not permitted to assume that the inorganic anions are without influence. On the contrary, HOFF-JÖRGENSEN has demonstrated that the solubility of Ca-pentaphytate is larger in 1-molar than in 0.2-molar NaCl,

which must be of importance in presence of calcium. MÖLLGAARD has stated that sulphuric acid has a retarding effect on phytase, but this statement is not valid, since it is drawn from three experiments with different flours, different amounts of added Ca, and different pH. Upon the whole, we cannot say that an acid has a retarding effect, we only can say that it activates more or less. To compare the effect of various organic acids, it would have given a better picture if equimolecular quantities had been used instead of the one percent solutions in HOFF-JÖRGENSEN—PORS-DAHLS experiments.

From what is said above, it is evident that the effect of an acid is dependent upon whether calcium is present or not. In doughs without added calcium, the quantity present will be small, in whole meal wheat, for instance, the ratio Ca : P is about 1 : 10, and this is too little to have any marked influence on the acid effect. Increasing amounts of added calcium, however, will change the picture, and these changes are subject of the following examinations.

Methods.

The doughs for examination were made in the farinograph with 300 gms of flour, 8 gms of yeast and 5 gms of salt, and sufficient water to give a consistency of 500 Brabender units. This is more correct than giving all doughs the same quantity of water, since the practical baker chooses the addition of water after the kind of the flour, until a definite stiffness of the dough is obtained. Besides, in the farinograph the dough at once gets the temperature wanted. CaCO_3 was added during mixing, in lots of 1.2 or 3.0 gms, corresponding to 0.4 and 1 per cent of the flour. The doughs were kept in a thermostat, and samples for analysis taken at suitable intervals. Acid solutions were added from a burette and washed out with part of the water.

For neutralisation of 1.2 or 3 gms of CaCO_3 should be required 24 or 60 ml of normal acid respectively. It appeared, however, that pH began to decrease when about half of these quantities were added, meaning that a sort of equilibrium is established between CaCO_3 , buffer substances and acid. Upon an addition of acid, pH was very rapidly balanced and kept constant during the whole experiment. The soluble Ca-salt first formed, will be precipitated as Ca-pentaphytate, but if pH gets below 5, its solubility will be markedly increased, making better conditions for

the phytase. On the other hand, the effect of the enzyme is restricted when pH gets below its optimum at 5.2—5.4, and the splitting process is the resultant of two opposite conditions:

Increased solubility of the substrate

Decreased activity of the enzyme.

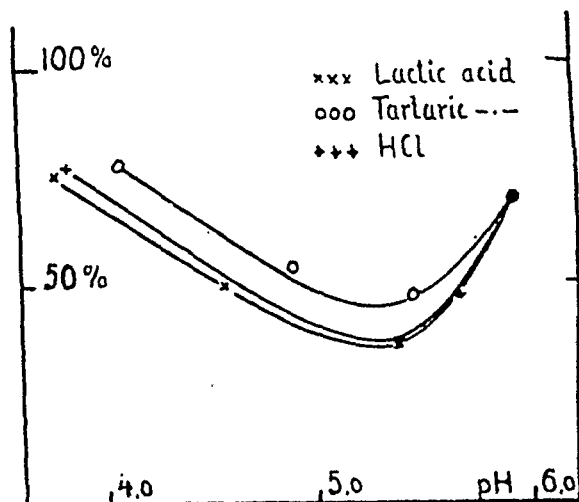


Figure 2. Influence of lactic, tartaric and hydrochloric acid anions on phytic acid decomposition. The graphs show remaining phytic acid P in percent of the originally present in whole wheat doughs after 3 hrs. at 27° C.

The more calcium present, the sourer the substrate must be to give sufficient soluble substance, and this explains both the smaller phytic acid decomposition with increasing amounts of Ca, and why the pH optimum is moved into a sourer direction. In addition to this fundamental process, then, comes the effect of the acid anion.

While the Danish investigators have operated with the same quantity of organic acids and adjusted pH by means of HCl or NaOH, the author has chosen to establish pH by means of the acid itself, because this is in better accordance with bakery practice. Thus, the sourer the dough, the more acid present. For the experiments, whole wheat meal was used, since this gave a suitable rate of decomposition.

a) *Effect of lactic, tartaric and hydrochloric acid in doughs without added calcium carbonate.*

The results, given graphically in Figure 2, show a better effect of lactic than of tartaric acid, in accordance with the findings of HOFF-JÖRGENSEN and PORSDAHL. Hydrochloric acid also seems very active. The pH optimum is about 5.3. The curves are not

symmetrical around this optimum, but show a greater activity on the sour side, because the anion effect is increased by the higher acid concentration. It will be noted that this is the case even for HCl, demonstrating an anion affect of this acid.

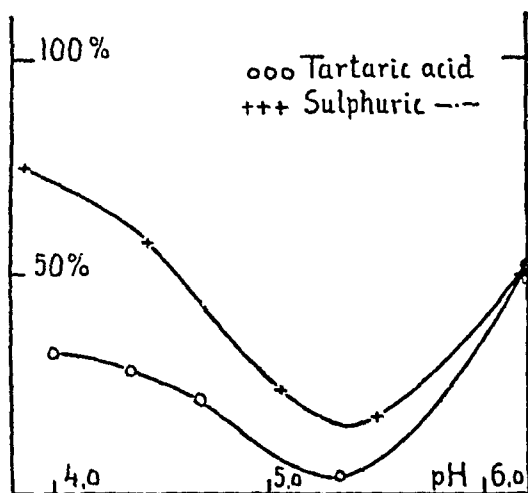


Figure 3. Effect of tartaric and sulphuric acid anions on phytic acid decomposition in whole wheat doughs without added CaCO_3 , showing remaining phytic acid P in per cent after 3 hrs. at 30°C .

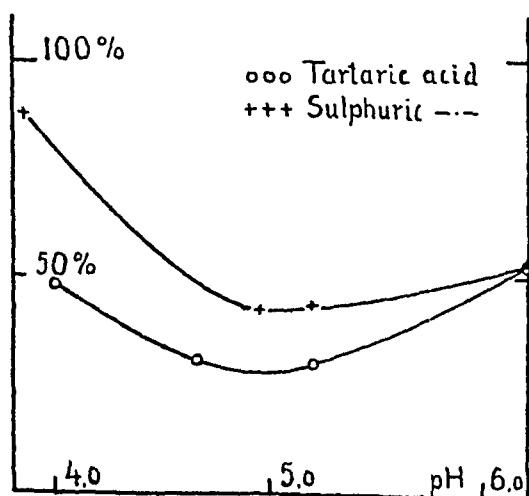


Figure 4. Same as Figure 3, but 0.4 % CaCO_3 added to the flour.

b) A comparison between tartaric and sulphuric acid in absence and presence of calcium carbonate. From Figure 3, it is seen that even without CaCO_3 tartaric acid is a better activator than sulphuric. With small quantities of acid, the pH effect is dominant, but with increasing amounts the different anion effect gets visible.

By addition of 0.4 % CaCO_3 (Figure 4) the decomposition rate is reduced for both, but most for sulphuric acid, and the optimal pH drops to 4.9.

Increasing the CaCO_3 addition to 1 % has no great further effect for tartaric acid, since the activating anion concentration is increased correspondingly, but for sulphuric acid the effect is deleterious (Figure 5).

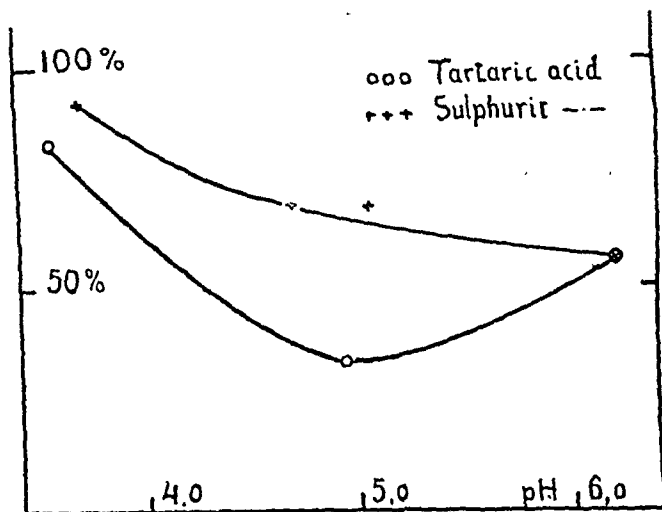


Figure 5. Same as Figure 3, but 1.0 % CaCO_3 added.

c) *Effect of neutralized acids in presence of CaCO_3 .*

The author's experiments may be compared with some of MÖLLGAARD's data, since the calcium addition is about 0.5 % for both series. This is done in table 1.

Table 1.

Effect of free and neutralized acids on phytic acid decomposition in presence of approximately 0,5 % Ca in the flour.

Dough nr.	Author	% Ca in flour	% acid	pH	Remaining Phytic acid P % 3 hrs.	Kind of acid	Notes
1.	M.	0.7	1.0	4.8	35	Tartaric	
2.	M.	0.5	1.0	5.9	21	»	Neutr. w. NaOH
3.	S.	0.45	1.85	4.9	16	»	
4.	S.	0.45	1.85	6.3	54	»	Neutr. w. NaOH
5.	S.	0.45	1.0	4.7	63	Sulphuric	
6.	S.	0.45	1.0	6.25	54	»	Neutr. w. NaOH
7.	S.	0.45	None	6.2	50.5	None	

Doughs nr. 1, 3 and 5, having about the same pH, show a good effect of tartaric, bad of sulphuric acid. At pH 5.9 MÖLLGAARD still finds an effect (dough nr. 2), but at 6.2 the author's doughs nr. 4 and 6 do not differ from the control nr. 7 without acids. This supports the author's view, that CaCO_3 has no effect when the medium is not sour enough to bring it into solution.

III. Contents of Phytic Acid P in ordinary Norwegian Bread Types.

Before the war four chief types of bread were usual in Norway, all of them made from flour of well-defined and constant extraction. These were white and dark bread of rye and wheat respectively. Whole meal was extracted to 92—94 % for both cereals, white rye to 67 and wheat flour to 73 %. Average composition for these flour types is given in table 2.

Table 2.

Contents of ash, total and phytic acid P, and Ca in in mgms pr 100 gms of dry matter.

Kind of flour	Degree of extraction	Ash	Total P	Phytic acid P	Ca
Wheat flour	73 %	550	125	38	23
White rye flour	67 %	870	168	100	26
Whole wheat meal	92—94 %	1,860	425	290	44
Whole rye meal	92—94 %	1,930	400	260	52

These percentages will, but for the contents of phytic acid P, be the same in dry matter of the bread (added NaCl not regarded). In eastern parts of the country, white wheat loaf was baked with skimmed milk instead of water. This meant an increase in dry matter of the bread of total P and Ca to 176 and 92 mgms.

During the baking process, the phytic acid contents will be reduced to various degrees, in accordance with the kind of flour and the influence of the baking scheme, of which the most important factors have been discussed before. These conditions, of course, will vary from one bakery to another, and, to get a true idea of the phytic acid contents of the bread types, numerous samples of bread were examined at different times. Now, the bread types of today are not quite the same as the normals before the war. The degree of extraction has been raised for the white flours,

Table 3.

Contents of total and phytic acid P in various bread types with data for their composition.

Date	Dry matter of crumb %	pH	In 100 gms of dry matter mgms		In the flour	
			Total P	Phytic acid P	Extr. %	Added CaCO ₃
1. <i>White wheat loaf.</i>						
³ / ₁₂ 1945	57.4	5.60	108	0	73	None
»	57.8	4.50!	—	0	73	÷
»	58.0	5.28	—	0	73	÷
»	57.0	6.00	180	0	73	÷
⁴ / ₇ 1946	56.2	5.85	240	28	80	0.4 %
»	57.3	5.00	290	0	80	»
2. <i>White rye bread.</i>						
²² / ₃ 1945	56.0	5.50	160	0	73	None
²⁴ / ₁₀ 1945	55.3	5.70	—	0	73	0.4 %
⁴ / ₁₂ 1945	55.3	5.55	—	0	73	»
»	54.9	5.48	—	0	73	»
»	55.1	4.81	200	0	73	»
»	55.1	5.72	—	0	73	»
3. <i>Whole wheat bread.</i>						
¹⁵ / ₁₂ 1942	54.7	5.80	463	256	92—94	None
⁷ / ₁ 1943	54.0	5.30	463	223	92—94	»
²² / ₁₀ 1945	55.2	4.80	415	120	92—94	»
²³ / ₁₀ 1945	54.5	5.20	415	215	92—94	»
²⁴ / ₁₀ 1945	—	5.45	415	240	92—94	»
²⁶ / ₁₀ 1945	54.5	5.55	415	285	92—94	»
⁵ / ₁₁ 1945	54.4	5.50	415	312	92—94	»
¹⁵ / ₁₁ 1945	54.0	5.50	415	276	92—94	»
¹⁸ / ₁₁ 1945	54.4	5.30	415	235	92—94	»
⁴ / ₇ 1946	54.8	5.83	510	220	92—94	0.4 %
»	54.9	5.95	415	175	92—94	»
¹⁵ / ₇ 1946	56.0	5.20	440	213	92—94	»
¹⁰ / ₁₂ 1946	58.0	5.70	440	220	92—94	»
»	56.2	5.90	400	207	92—94	»
4. <i>Whole rye bread.</i>						
³ / ₁₂ 1945	54.5	4.67	367	32	92—94	None
»	—	—	—	0	92—94	»
»	—	—	—	0	92—94	»
⁴ / ₇ 1946	54.4	4.42	332	108	92—94	»
»	52.1	4.88	420	170	92—94	0.4 %
¹⁵ / ₇ 1946	55.7	5.52	380	—	92—94	»
»	53.5	5.00	375	130	92—94	»
“Knekkebrød”:						
Grey type	91.5	6.00	372	225	92—94	?
Brown »	91.5	5.76	372	150	92—94	?
“Flatbrød”.						
Commercial	93.5	5.80	325	93	?	None
Whole barley from Valle, Setesdal ...	92.3	5.30	382	212	?	None

and the addition of CaCO_3 extended to all types. Notes for such alterations have been given in table 3, where the results of the examinations are reproduced.

For white wheat bread, the degree of extraction first was the same as before the war. Later, it has been raised to 80 %, but even here little or no phytic acid was found. The dough liquid has been water. In a baking experiment (the milk situation does not as yet permit use of skimmilk in commercial bread) was found 55 mgms of phytic acid P in the milk loaf, none in the water loaf from the same flour. This may indicate a retarding effect of the calcium salts from the milk, but, as these raise the total calcium contents to 100 mgms, there will be a fair excess even if all phytic acid is precipitated. From nutritional reasons, then, it is highly advisable to bake this bread with milk, when available.

This procedure, however, cannot be recommended for whole wheat bread because of its high contents of phytic acid.

The normal household bread from white rye flour, usually containing 15—30 % wheat, has proved free of phytic acid in all cases examined, although 0.4 % CaCO_3 was added to the flour, and the degree of extraction was a little elevated (73 % against 67 % before the war.) For provisional reasons, this flour type has not as yet been established. At present, January 1947, it contains equal parts of rye and wheat extracted to 80 %, but even now little or no phytic acid has been found in the bread. Consequently, when this higher extraction holds, we can feel certain that the normal bread of 67 %-extracted flour will always be free of phytic acid.

In whole rye bread, the contents of phytic acid can vary rather much, some samples showing nothing at all, while others may contain considerable quantities. Because of the high phytase activity of whole rye, it should not be difficult to bake this bread free of phytic acid. However, this bread makes but a small part of the total consumption, and so the case with the hard types, "knekkebröd" and "flatbröd", where the contents of phytic acid usually are high. It should be noted that "knekkebröd" appears in two types, one brown, fermented with yeast, and one greyish white, leavened with air which is whipped in by low temperature, the dough being cooled with ice. In this case, phytase activity obviously must be low, and correspondingly much phytic acid is found. Swedish and Danish examinations confirm the author's

findings, that in grey "knekkebrød" about $\frac{2}{3}$ of the total is present as phytic acid P, against $\frac{1}{3}$ in the brown type, where dough temperature is more normal.

It remains to make a review of the physiological importance of these findings. If phytic acid in the intestine is precipitated as penta-Ca-phytate, 6P corresponding to 5Ca, 1 gm of phytic acid P should precipitate 1.075 gms Ca. For instance, in oatmeal with an average of 195 mgms of phytic acid P, theoretically 317 mgms Ca might be precipitated, or, since oatmeal contains only 57 mgms, not less than 260 mgms might be precipitated from other foods if present. This gives the interesting consequence, that if oat porridge is eaten with 200 ml milk containing 240 mgms Ca, this calcium should be completely lost, unless the milk is taken some time before or after the porridge! For this reason it has been advised to improve oat products by addition of 1 % CaHPO_4 or CaCO_3 , giving sufficient Ca for immobilization of the phytic acid.

We can assume, however, that these theoretical losses will never be reached. Apart from the possibility, that some of the Ca-phytate may be utilized, which is not yet finally decided, another moment may play an important part, namely the presence of magnesium in the food. Phytic acid is liable to form heavily soluble Ca—Mg double salts, in fact, such compounds form a great part of the natural "phytin" in plants. It has not been studied what compounds will be precipitated with different proportions Ca : Mg in solutions, and not the relative solubility of these compounds by varied hydrogen ion concentrations. In whole wheat, Mg dominates over Ca in a proportion 4 : 1, and it is permitted to expect that this will influence upon the precipitation of Ca in the intestine. On the other hand, the clinical investigations of HARRISON and MELLANBY, McCANCE and WIDDOWSON and of ANDERSEN and HOFF-JÖRGENSEN, give evidence of a reduced utilization of Ca and Fe on a food rich in phytic acid, and this at any rate makes it desirable to have this compound removed from the food as far as possible.

From the important Norwegian bread types only the whole wheat bread, Kneip- or Graham bread presents a steady appearance of greater quantities of phytic acid. This fact deserves consideration, since this bread is specially recommended by some health propagandists because of its high contents of minerals and vitamins, and forms an important part of the so called Oslo

breakfast. It can be stated now that the value of the minerals is overrated. In this connection, a warning must be given against the recommendations of certain physicians, of giving babies porridge of whole meal wheat. In porridge cooking, namely, the phytase activity has no chance at all, and the full amount of phytic acid comes into the food.

In whole wheat, the phytase activity is much lower than in rye, and even if something can be obtained by proper measures in a bakery under control of a modern laboratory, we never can expect to bake whole wheat bread free of phytic acid.

During the war, the 95 %-extracted meal contained, at times, large quantities of oats and barley, and had an addition of 0.4 % CaCO_3 in order to give the population a source of calcium as a substitute for the milk. The value of such a step, which may seem doubtful, will be discussed in another publication. After the war, this addition has been extended to all flour types, dark and white. The author's examinations have shown that CaCO_3 in a dough does not impair the phytase activity so long as it is not dissolved, but when this takes place, that is on the first appearance of acidity, the phytic acid decomposition will be retarded. Consequently, the more calcium present, the more phytic acid will be left intact, and the more Ca-phytate formed. The question then arises, shall we add calcium salts to precipitate the phytic acid, or should we avoid calcium to obtain a better phytic acid decomposition? So much is clear, that a calcium addition is much more valuable in white than in whole meal flours, and consequently, as long as an addition of calcium to our food is found necessary, the author will recommend that it be limited to white flours only.

Acknowledgement: The author is indebted to J. L. NERLIEN's fond for economical support.

Summary.

Factors influencing phytic acid decomposition during baking are type of cereal, degree of flour extraction, temperature, acidity and moisture contents of the dough, and dough scheme. The effect of some acid anions in presence and absence of calcium carbonate is studied. Without Ca, lactic and hydrochloric acid proved most effective. With Ca, tartaric and citric acid are better

because of their complex-forming capacity, and sulphuric acid worse the more Ca is present. As a whole, increasing amounts of Ca will retard phytic acid decomposition, but an addition of CaCO_3 is not harmful unless the dough is sour enough to bring it into solution.

Data on the contents of phytic acid in ordinary Norwegian bread show that among the more important types, only whole wheat bread, "kneipbrød", regularly contains appreciable quantities. The value of this bread as a mineral source, then, is over-rated because of the precipitation of Ca and Fe in the intestine in presence of much phytic acid. This precipitation, however, probably will not obtain its theoretical value, because Mg, which has a great overweight over Ca in wholemeal bread, is likely to take the place of some Ca in formation of insoluble double salts with phytic acid.

After all, the value of adding calcium carbonate to whole meal flour in order to make it a source of calcium seems doubtful. If such a gift of calcium still is found necessary, it will be more rational to limit it to white flours, from which the bread practically is free of phytic acid.

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Histamine and Reactive Hyperaemia.

By

KERSTIN EMMELIN and NILS EMMELIN.

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The question whether histamine plays a part in reactive hyperaemia still remains unsettled. Whereas BARSOUM and GADDUM (1935), BARSOUM and SMIRK (1936) and ANREP et. al. (1944) found an increase in the histamine content of the venous blood or plasma emerging from a limb during reactive hyperaemia, after a period of circulatory obstruction, other investigators were not able to find such an increase (KWIATKOWSKY 1941, EMMELIN, KAHLSON and WICKSELL 1941). The negative results, however, do not justify the conclusion that histamine is not the agent responsible for the vasodilatation of reactive hyperaemia. Histamine accumulated in the tissues during the circulatory arrest, might be inactivated locally or removed by the lymph. Even if it escapes into the blood stream the possibility remains that the rise in histamine concentration of the venous blood, collected from a limb during reactive hyperaemia, is too small to be detected with the methods available. It is well known that histamine injected intravenously can elicit vascular responses without causing an increase in the blood histamine concentration great enough to be detectable in a blood sample, extracted and tested on the isolated guinea-pig gut in the usual way (ROSE 1940, EMMELIN, KAHLSON and WICKSELL 1941).

The antihistamine substance β -dimethyl-aminoethyl benzhydryl ether hydrochloride (LOEW et al. 1945) is known strongly to antagonise the action of histamine on the arterial blood pressure (WELLS et al. 1945, LOEW et al. 1946). By using this drug

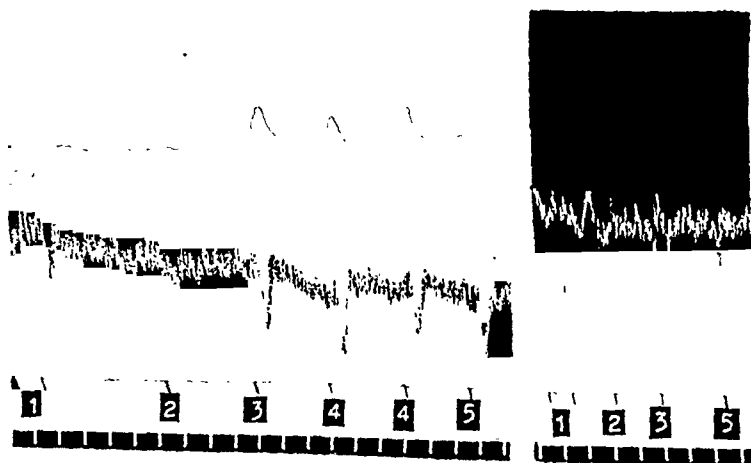
it may be possible to tackle the problem of a relation of histamine to reactive hyperaemia in another way.

Methods.

The experiments were performed on cats under chloralose. The blood pressure in the carotid artery was measured by means of a mercury manometer. The volume of one hindleg was registered by using a plethysmograph described by DALE and RICHARDS (1918). The iliac artery of the limb was freed close to the aorta and other arteries to the leg were tied. Circulatory arrest was brought about by putting a clamp on the iliac artery. Intraarterial injections of histamine and acetylcholine were made through a cannula introduced into the central stump of the iliac artery of the other leg close to the aorta. β -dimethyl-aminoethyl benzhydryl ether hydrochloride¹ was given intravenously in a dose of 10 mg/kg of body weight.

Results.

Eight experiments were made giving concordant results. The figure shows a typical experiment. Arterial obstruction during one minute (at 1) is followed by a marked increase of the leg volume. Intraarterial injections of histamine (0.005 γ at 3 and 0.001 γ at 4) and of acetylcholine (0.005 γ at 5) cause similar



Cat under chloralose. Records from above: volume of left hindleg, blood pressure, signal, time in minutes. 1) arterial obstruction during 1 minute, 2) 0.4 ml Tyrode solution injected into the iliac artery, 3) 0.005 γ histamine, 4) 0.001 γ histamine, 5) 0.005 γ acetylcholine. Between the two sections of the tracing intravenous injection of 10 mg/kg deseryl.

¹. In these experiments "Deseryl Leo", supplied by courtesy of AB Leo, Helsingborg, was used.

effects whereas the corresponding volume of pure Tyrode solution (0.4 ml at 2) scarcely has any effect. Deseryl given intravenously causes only a very transient fall in blood pressure if injected slowly. After deseryl circulatory arrest is still followed by marked vasodilatation. Histamine on the other hand gives only a small response. From the figure it can be seen that 0.005 γ histamine elicits a much smaller effect after deseryl than did 0.001 γ before administration of the antihistamine substance. The action of acetylcholine is not much decreased by deseryl. It may be observed that the action on the arterial blood pressure of both histamine and acetylcholine given intraarterially into the leg is reduced after deseryl whereas reactive hyperaemia is still accompanied by a fall in blood pressure.

Discussion and Summary.

The experiments show that β -dimethyl-aminoethyl benzhydryl ether hydrochloride markedly reduces the effect of intraarterially injected histamine on the vessels of the cat's hindleg. The antihistamine substance does, however, not diminish the vasodilatation following circulatory obstruction. Thus the experiments do not support the view that histamine is responsible for the vasodilatation of reactive hyperaemia. The possibility remains that the drug may abolish the effect of histamine, reaching the vessels by the blood stream, but not that of histamine, released into the tissue spaces. Against this objection it may, however, be argued that deseryl diminishes the response of the skin vessels to intracutaneously injected histamine (GILJE 1947).

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The Toxicity of a Dialyzed Casein Digest.

By

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Amino acids, prepared by enzymatic hydrolysis of protein, especially casein, have been used in medicine and surgery during the last few years for conditions of protein lack.

In Sweden a mixture of amino acids, »Aminosol», prepared by WRETLIND (1944), has been used, both orally and parenterally. The product is made by digestion of pure casein with trypsin and erepsin. After hydrolysis the digestion mixture is dialyzed, by which a preparation is achieved, containing pure amino acids and a smaller part of low molecular peptides. High molecular peptides and proteins do not pass the dialyzing membrane.

As the need for protein is large in several pathological conditions it is desirable in some cases to be able to administer as much as 200 g protein a day or more. WRETLIND (1944) has given 500 ml 3.3 % Aminosol intravenously to a rabbit weighing 2 kg with no ill effect. If it could be assumed that the toxicity in man corresponds to that in rabbit, this dose would imply 17.5 l 3.3 % Aminosol or 578 g amino acids. A more exact knowledge of the toxicity of amino acids or mixtures of amino acids is essential, and the aim of this work is to determine the toxicity of this enzyme casein hydrolysate.

Products tested.

For determination of the toxicity a dialyzed casein digest (»Aminosol»),¹ prepared according to WRETLIND (1944), has been used. Products intended for oral and parenteral use have been tested.

¹ Manufactured and placed at our disposal by Vitrum, Stockholm.

The product for oral use contained 12.0 % nitrogen and the product for intravenous use 12.7 % nitrogen, calculated on dry substance, of which 76 and 67 % respectively are found as free amino acids and the rest as peptidebound nitrogen (WRETLIND 1947). The following amino acids are found in free form:

alanine, arginine, asparaginic acid, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine, valine.

Some of these amino acids are found as sodium salts and thus the solutions had a pH of 7.0—7.3.

Orally: Aminosol dry powder (12.0 % nitrogen) was suspended in distilled water to a concentration of 20—40 % (20, resp. 40 g per 100 ml suspension).

Parenterally: a. The ordinary 3.3 % Aminosol solution (0.42 % nitrogen) for intravenous use was injected in some preparatory experiments.

b. In order to get a higher concentration the 3.3 % Aminosol solution was concentrated in vacuo to 10 % (1.27 % nitrogen), this solution used in some preparatory experiments.

c. For use throughout the experiments the Aminosol was concentrated in vacuo to 26 % (3.29 % nitrogen).

Experimental.

A. Oral toxicity.

The oral toxicity was determined in 15 rabbits, 2.2—4.2 kg, by administering the Aminosol suspension by stomach tube, in doses from 10 to 20 g per kg, the whole dose was given within 5 minutes.

In order to be able to administer lethal doses with a reasonable quantity of solution we used concentrations between 20 and 40 %; thus a very hypertonic suspension — 2.5 % solution being isotonic with the blood.

The animals who died showed a distended abdomen and a heavy dyspnea. On postmortem examination the bowels were heavily distended and filled with a great quantity of a light-brownish liquid with the characteristic smell of Aminosol. These findings speak in favour of the assumption that water to a considerable degree has passed over to the intestines from the rest of the body.

The results of these experiments are shown in table 1.

It is evident from these experiments that the lethal dose (LD_{50}) lies between 12.5 and 15 g per kg body weight with a 20—40 % suspension.

Table 1.

Oral Administration of Aminosol in rabbits.

Dose		Number of animals	Mortality.
Conc. %	g/kg		
28 40	10 10	1 2	0/1 0/2
20 40	12.5 12.5	1 3	0/1 1/3
20 40	15 15	1 4	1/1 4/4
40	20	3	3/3

B. Parenteral toxicity.

Mice. Aminosol was administered subcutaneously to white mice, mainly males, weighing 11–22 g, on an average 15–18 g, 134 animals in all.

A 3.3 % solution, administered in doses of up to 0.15 ml per g, corresponding to 10.5 lit. in a 70 kg person, proved to be non-toxic. The animals only showed a slight decrease in spontaneous activity.

A concentration of 10 %, when administered in doses of more than 0.1 ml/g, proved to be fatal. Before exitus which came after 1–4 hours, convulsions, loss of righting reflexes and lateral position occurred. The death was due to respiratory and cardiac standstill.

The lethal dose was determined by administration of a 26 % solution to 110 animals in all, in doses of 0.05 to 0.15 ml/g. The LD_{50} was determined graphically after transformation of percentage mortality to probits according to BLISS (1938), and plotting the probit values against log dose, which gave a rectilinear relation (fig. 1.) LD_{50} was 20.2 g/kg, and the standard deviation 30.0 %. The convulsion dose was approximately 10 g/kg.

Guinea-pigs. The intravenous toxicity was assessed by slow intravenous injection in guinea-pigs, following the KNAFFL-LENZ (1926) technique for assaying cardiac glucosides according to the principles, stressed by GOLDBERG (1942)

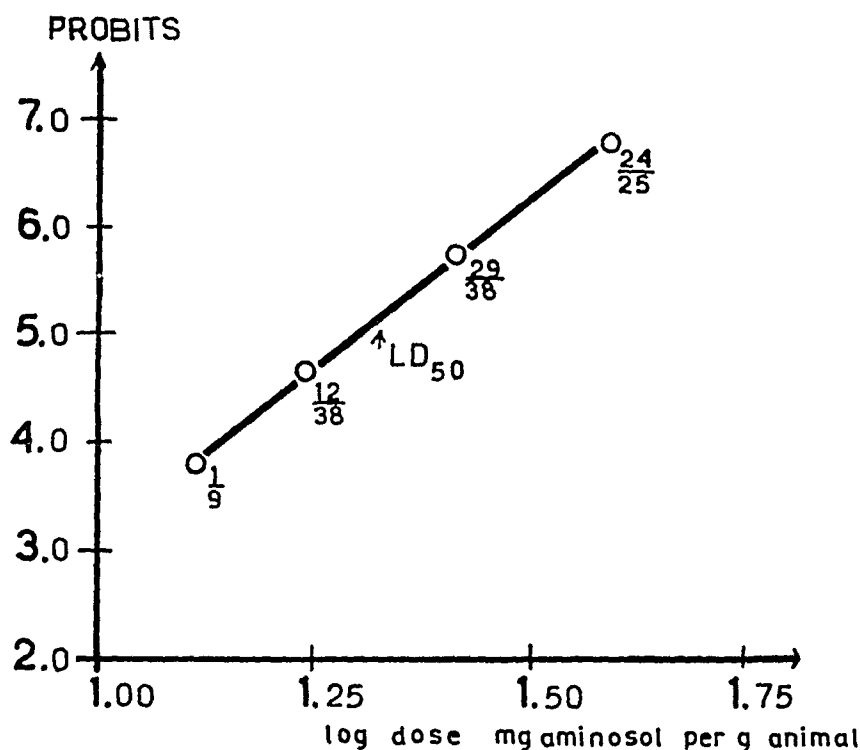


Fig. 1. Dose-Mortality Curve of Aminosal in Mice. Number of animals at each dosage and LD_{50} , at the arrow. Abscissa: Log dose mg Aminosal per g animal subcut. in 26 % sol. Ordinate: Probits.

The animals, weighing 400—530 g, 10 animals in all, were anesthetized by subcutaneous injection of 1.75 g urethan per kg body weight in 20 % solution. After 1—2 hours the trachea and one jugular vein were cannulated, and the Aminosal solution in a concentration of 26 % was slowly injected at a constant rate of 1.5 ml per minute. When the respiration ceased, the chest was opened, artificial ventilation set in, and the injection was continued at a rate of 0.5 ml per minute, until the heart definitely stopped.

The respiratory standstill occurred with this technique after a mean time of 17 minutes, and the mean dose was 56.4 cc/kg, corresponding to 14.6 g/kg (table 2).

The cardiac standstill occurred after 21 minutes on an average and the lethal dose was 66.5 ml/kg, corresponding to 17.3 g/kg.

Rabbits:

A. No anesthesia. Injection of 10 ml/kg of a 26 % solution, corresponding to 2.6 g/kg, into the marginal vein of the ear at a

Table 2.

*Intravenous Administration of Aminosol in Anesthetized Guinea-Pigs.
Conc. 26 %, Injection Rate 3.3 ml/min/kg, Anesthesia
1.75 g urethan per kg.*

Nr.	Weight kg	Injection Time min		Volume per kg ml	
		Resp. Standstill	Cardiac Standstill	Resp. Standstill	Cardiac Standstill
1	0.500	19	19	60.0	60.0
2	0.420	11	17	52.4	70.2
3	0.470	17	17	58.5	58.5
4	0.440	17	22	58.0	71.5
5	0.530	17	20	45.3	49.0
6	0.400	16	27	60.0	86.1
7	0.480	21	26	62.5	75.0
8	0.490	20	24	61.1	65.4
9	0.465	19	28	58.0	81.7
10	0.440	14	14	47.7	47.7
Mean:		17.1	21.2	56.4	66.5

constant rate of 3 ml/min in 5 rabbits, weighing 1.0—3.1 kg, brought about only slight symptoms: one animal showed some loss of spontaneous activity, a slight change in righting reflexes, an increase of respiration, and the symptoms had passed in 1—2 hours. The other animals showed no symptoms. No fatalities.

Injection of 15 ml/kg in 26 % solution in 5 animals, 1.8—3.1 kg, at a rate of 3 ml/min, brought about the same symptoms in somewhat heavier form in all animals, no fatalities.

Injection of 25 ml/kg of a 26 % solution in 6 animals, 1.7—2.5 kg, proved to be fatal in 3 animals out of 6, the death occurring within 6—24 hrs, and preceded by total loss of righting reflexes, running movements in the limbs and shallow respiration. The other animals recovered.

B. Anesthesia. The acute intravenous lethal dose was determined by slow injection of 26 % Aminosol into a jugular vein at a constant rate of 3 ml/min, after anesthetizing the animals with 1.4 g—1.6 g urethan per kg intravenously in 20 % solution and then proceeding like in guinea-pigs. The respiration standstill occurred after 46 minutes, and the dose was 71.4 ml/kg, corresponding to 18.5 g/kg.

Table 3.

Intravenous Administration of Aminosol in Anesthetized Rabbits.
Conc. 26 %, Injection Rate 1.5 ml/min/kg, Anesthesia 1.4—1.6 g
urethan per kg

Number of animal	Weight kg	Injection Time min		Volume per kg ml	
		Resp. Standstill	Cardiac Standstill	Resp. Standstill	Cardiac Standstill
1	1.960	53	64	82.5	99.2
2	2.070	50	64	72.5	87.9
3	1.720	25	30	43.6	49.7
4	1.870	55	83	87.1	133.0
5	2.170	—	70	—	96.8
Mean:		46	62	71.4	93.8

The cardiac standstill occurred after 62 minutes, and the lethal dose was 93.3 ml/kg, corresponding to 24.2 g/kg. (Table 3.)

Discussion.

When trying to calculate the toxicity in man from the values found in animals, the following facts must be borne in mind: The tolerance of mice is generally higher than that of man, whereas the tolerance of guinea-pigs or rabbits corresponds more to that in man. Further it is known that the toxicity of many substances increases with increasing concentration, which has been shown among others for local anesthetics (GOLDBERG, to be published).

This seems to be true also for Aminosol, as it was impossible to produce toxic symptoms by a 3.3 % solution in a rabbit (WRETILIND 1944), when given up to 8.25 g/kg, but in our experiments a 26 % solution proved to be lethal at a dose of 6.5 g/kg. Finally it must be stressed that the intravenous toxicity depends also on the injection rate.

As to the oral toxicity it must be emphasized, that the real toxicity most probably is lower than our values found, because we had to use very hypertonic solutions, 20—40 %, and the total volume administered was as much as 50—100 ml per kg.

The conclusions from our experiments are given in table 4, from which will be seen that the lethal dose for man for intravenous administration will be approximately 14 lit. of a 3.3 % solution

Table 4.

Toxicity of Aminosol in Animals, and Calculated Toxicity in Man.

Animal	Number of Animals	Administration	Conc.	Injection Rate ml/min	Lethal Dose g/kg	Corresponding Lethal Dose in Man, weighing 70 kg		
						3.3 % Aminosol		
						Amino Acids g	Injection Rate lit/min	Total Amount lit.
Mice	110	Subcutaneous	26 %	—	20.2	1,410	—	42
Guinea-Pig	10	Intravenous (Anesthesia)	26 %	3.3	17.3	1,210	0.23	36
Rabbit	15	Oral	20-40 %	—	12.5-15	875-1,050	—	27-32
	6	Intravenous	26 %	1.5	6.5	445	0.11	14
	5	Intravenous (Anesthesia)	26 %	1.5	24.2	1,690	0.11	52

and for oral use 875—1050 g amino acids. These figures refer to a single dose, and it can be assumed that if these quantities are distributed over a whole day the lethal dose must be higher.

Summary.

The oral, subcutaneous and intravenous toxicity of Aminosol, an enzymatic casein digest, has been determined in mice, guinea-pigs and rabbits. The lethal dose was found to be:

1. for mice 20.2 g/kg, administered subcutaneously in 26 % solution;
2. for rabbits 6.5 g/kg, administered intravenously in 26 % solution;
3. for rabbits 12.5—15 g/kg, when given orally in 40 % suspension;
4. in guinea-pigs, using the KNAFFL-LENZ technique, the respiratory standstill occurred after 14.7 g/kg, and cardiac standstill after 17.3 g/kg;
5. in rabbits, using the same technique, the respiratory stand-

still occurred after 18.5 g/kg, and the cardiac standstill after 24.2 g/kg.

It could be assumed that this would correspond in man to an oral, single dose of approximately 875—1050 g amino acids, and intravenously to 14 liters of a 3.3 % solution.

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Contributions to the Understanding of the Regulation of the Gastric Acidity.

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On the subject of the regulation of the secretion of gastric juice much work has been performed, judging from the extensive literature existing, but opinions still differ more on this point than on any other point within the physiology of gastric secretion. The interest in the subject is quite natural, as the understanding of the regulation of the acidity has not only importance theoretically, but also clinically in the treatment of peptic ulcer and related diseases, where the acidity of the gastric juice is considered important for the origin of the disease.

The problem of the acidity of the gastric juice at the moment of secretion is much investigated, but it can hardly be said that any acknowledged solution has been arrived at as yet. According to one opinion the gastric juice is always secreted with a constant, maximal acidity. When different acidities are found during the course of secretion, this should be due to the gastric juice being mixed with mucus from the gastric mucosa, diluting, neutralizing or absorbing the acid. The greater the rate of secretion the less the effect of the mucus and the higher the acidity. This presumption, first set forth by HEIDENHAIN, was accepted by PAVLOV and his school and seems subsequently to be the one most commonly adopted (HOLLANDER and COWGILL, TEORELL, IHRE, BOLTON and GOODHEART).

Another explanation was given by ROSEMAN, who was of the opinion that the gastric juice is secreted with a fairly constant

chloride concentration, whereas a varying concentration of the hydrogen ions in relation to the remaining kations, especially Na^+ , causes the observed changes of acidity.

BOLDYREFF maintains that the principal reason for acidity decrease is regurgitation of duodenal juice into the stomach. A number of authors are of the opinion that a dilution takes place with a neutral or alkaline fluid, which together with the hydrochloric acid should be secreted from the gastric mucosa (KATSCH and KALK, LIM, McLAGAN). BABKIN stresses that the stomach mucosa is composed of various glandular structures and that the qualitative changes in the composition of the gastric juice may depend not only on the qualitative changes, but also on the quantitative changes in the activity of separate groups of cellular elements in a given gland.

Finally, TEORELL has put forward another explanation regarding the acidity fluctuations, namely that the gastric juice is secreted as practically pure HCl , isotonic with blood, and this is followed by a diffusion outwards through the gastric mucosa of H^+ ions, and an inward diffusion of Na^+ ions. The larger the amount of fluid, the slower the fall of acidity. TEORELL points out, however, that several of the previously mentioned factors probably are assisting in the acidity regulation.

According to ALVAREZ, cytological-chemical researches of the gastric cells at the moment of secretion have shown that the oxyntic cells secrete a gummy substance, probably a protein hydrochloride, which, passing through the lumen of the glandular tubules, is broken down, forming hydrochloric acid. The cytoplasm of the oxyntic cells remains during the entire process of secretion practically of normal reaction or at least considerably less acid than the gastric juice (according to DAWSON and IYR the pH is between 6.8 and 3.0). Nor do chlorides concentrate in the oxyntic cells, whereas chlorides are said to accumulate in the tissues surrounding the gastric glands.

Own Investigations.

The present investigations have been based upon examination of samples of gastric juice obtained from human individuals by means of intravenous injection of insulin. A thin rubber tube is introduced into the stomach, and the resting gastric contents are aspirated, whereafter 20 i. u. of insulin are injected intravenously. The stomach is completely emptied every 15—20 minutes. Approximately 30 minutes

after the injection a vigorous increase of the volume and acidity of the gastric juice is observed, and considerable amounts of juice can as a rule be aspirated during the following 60—90 minutes. The samples collected are examined for 1) volume; 2) acidity, by titration with $n/10$ NaOH, using dimethyl-diamido-azo-benzol (Töpfers reagent) as indicator; 3) chlorides, determined by the method of Volhard; 4) pepsin, ad modum Mett.

The samples have been taken partly from persons without gastric disease, partly from persons with gastric diseases, such as peptic ulcer or gastritis, the two groups comprising 33 and 54 persons, respectively. For comparison a series of samples of fasting gastric juice has been collected from a number of these persons without stimulation of any kind. The tests include 172 samples of fasting gastric juice without stimulation and 365 samples after stimulation with insulin.

Relation between Acidity and Chlorides of the Gastric Juice.

The question arises whether the acidity and the chlorides of the gastric juice are functions of one another, or on the contrary independent of one another. In order to solve this problem the results from all samples of juice have been arranged according to acidity, placing all samples with acidity 1—9 m.eq./liter in one group, samples with acidity 10—19 m.eq./liter in the next, 20—29 m.eq./liter in the following, etc. The group with acidity 0 is taken separately. An average figure for rate of secretion, acidity, chloride and pepsin is computed for each group.

In Table 1 the results are given of these average computations for all groups of juice samples after stimulation with insulin, together with the standard errors of the acidity and chloride means and the standard deviations of the chlorides. Results of the 172 samples of fasting secretion are computed and tabulated in the same way. The fasting juice has been obtained fractionally, so that the time during which each sample of juice has been secreted is known, thus enabling the rate of secretion to be determined.

It will be seen from the table that a close relation exists between acidity and chloride. Increasing acidity corresponds to increasing chloride. Calculation of mean error and standard deviation shows rather narrow limits for each particular group. At acidity 0 an average chloride value of 70 ± 4.8 is found, together with a standard deviation of 20, *i. e.* $2/3$ of the chloride values can be expected within 50 and 90 m.eq./liter. At higher acidities more narrow limits are found. The group with acidity between 60 and

69 shows an average chloride of 114 ± 2.2 and standard deviation ± 10 , whereas the group with acidity 100—109 shows an average chloride value of 135 ± 0.9 with standard deviation of ± 5 .

The neutral chlorides show a distinct decreasing tendency from the average of 70 m.eq./liter at the acidity 0 to app. 20 m.eq./liter at the highest acidities.

Contrary to this the rate of secretion only shows little parallelism to the acidity; a tendency, however, is noted to increased rate of secretion with rising acidity. On an average app. 1 ccm/min. is secreted at the lower acidities, 2 ccm/min. at the higher.

The pepsin activity shows an increasing tendency when the acidity rises from 0 to moderate values. At higher acidity there is apparently no parallelism between pepsin and acidity. At acidity 0 an average pepsin value of 52 is found, whereas at acidities of 80, or more, a pepsin value of 200, or more, is found (measured by the square of the lengths of the digested protein columns.)

Comparing gastric juice obtained by insulin stimulation to juice produced without stimulation (Table 1), a close concordance will be noted. However, acidities above 70 to 80 m.eq./liter are seldom met with without stimulation, while insulin stimulation frequently brings about acidities in excess of 100 m.eq./liter, the highest found being 142 m.eq./liter. The groups, for which a comparison can be made, *i. e.* groups with acidity from 0—80, are, however, nearly identical in regard to values of total chloride, neutral chloride, rate of secretion and pepsin, no deviation considerably exceeding that to be expected according to the standard error of the means.

From this it may be seen that juice after insulin stimulation does not show any essential difference from fasting juice, apart from the fact that, after insulin, samples of considerably higher acidity, chloride and pepsin content can be collected than without stimulation. Insulin juice of lower acidity is entirely like secretion without stimulation, the higher acidities constituting an extension of the series of acidity values.

On the diagrams (Fig. 1 and 2) the curves represent corresponding figures of acidity — total chloride and acidity — neutral chloride, respectively. It will be seen that these curves are nearly straight lines as to the insulin tests as well as to tests with gastric juice without stimulation, and that these two sets of curves are very similar.

Table 1.
Secretion after stimulation by insulin.

Group of acidity	Number of samples	Volume of secretion (cem/min.)	Mean of		Neutral chloride	Pepsin mm ²
			Acidity with mean error	Total chloride with mean error		
0	17	1.2	0	70±4.8	70	52
1—9	7	1.2	6±0.3	80±5.9	74	65
10—19	24	1.4	15±0.5	85±4.0	70	142
20—29	28	1.2	24±0.5	94±2.7	70	135
30—39	29	1.4	35±0.4	105±2.4	70	155
40—49	26	1.5	44±0.5	104±2.6	60	188
50—59	30	1.5	54±0.5	112±1.6	58	130
60—69	22	1.4	64±0.6	114±2.2	50	166
70—79	24	2.0	74±0.6	121±1.5	47	184
80—89	30	2.5	84±0.6	125±1.3	41	199
90—99	37	1.9	94±0.5	132±0.8	38	214
100—109	29	1.9	105±0.5	135±0.9	30	186
110—119	26	2.3	114±0.5	141±1.1	27	225
120—129	20	1.8	123±0.6	146±1.1	23	255
130—139	12	2.5	134±0.7	152±1.1	18	227
140—149	4	3.2	141±0.6	160±2.2	19	190
Fasting secretion						
0	26	1.0	0	78	78	43
1—9	19	0.7	6	83	77	94
10—19	17	1.1	14	88	74	109
20—29	23	1.6	23	87	64	128
30—39	26	1.1	33	86	53	150
40—49	9	1.3	46	101	55	174
50—59	18	1.9	55	109	54	133
60—69	19	1.1	64	114	50	139
70—79	8	1.5	74	114	40	165
80—89	6	1.6	82	123	41	142
90—99	1	1.3	94	139	45	196

It is of interest to calculate the exact, mathematical expression for the straight lines best covering the curves found. For the establishments of these expressions the results found by the insulin tests are used, as these are based on the largest number of individual results. The equation of regression for these values are: $m = 1.73 k - 133$, m standing for acidity and k for the total chlorides. This line intersects the horizontal axis at the point $m = 0$, $k = 77$. Further it is of importance to compute the point where $m = k$, *i. e.* acidity = total chloride. This must take place at the theoretical acidity maximum, namely when all chloride appears as acid chloride. Calculation shows that this takes place at the point $m = k = 182$.

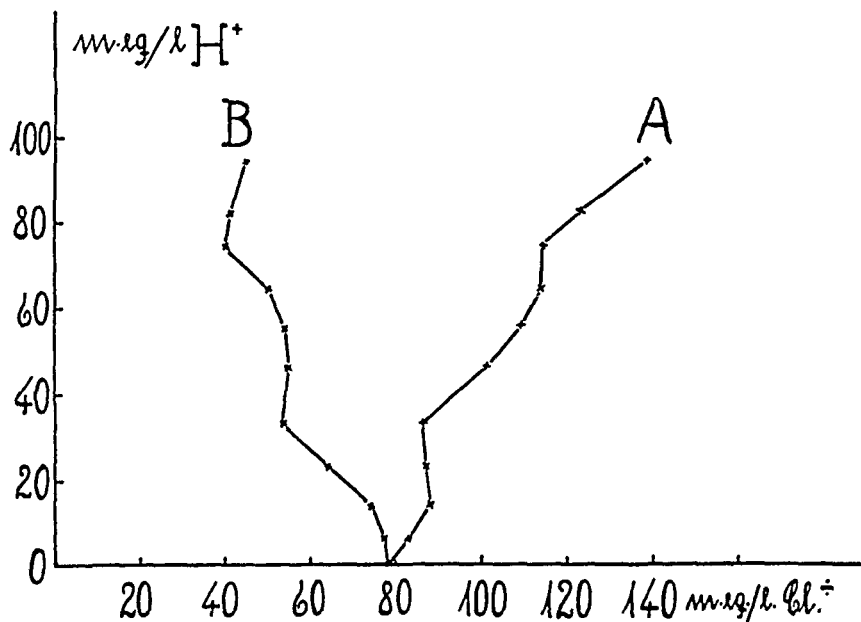


Fig. 1. Fasting secretion. Relation between acidity and chloride.

Abscissa: Chloride. Ordinate: Acidity.
A: Total chloride. B: Neutral chloride.

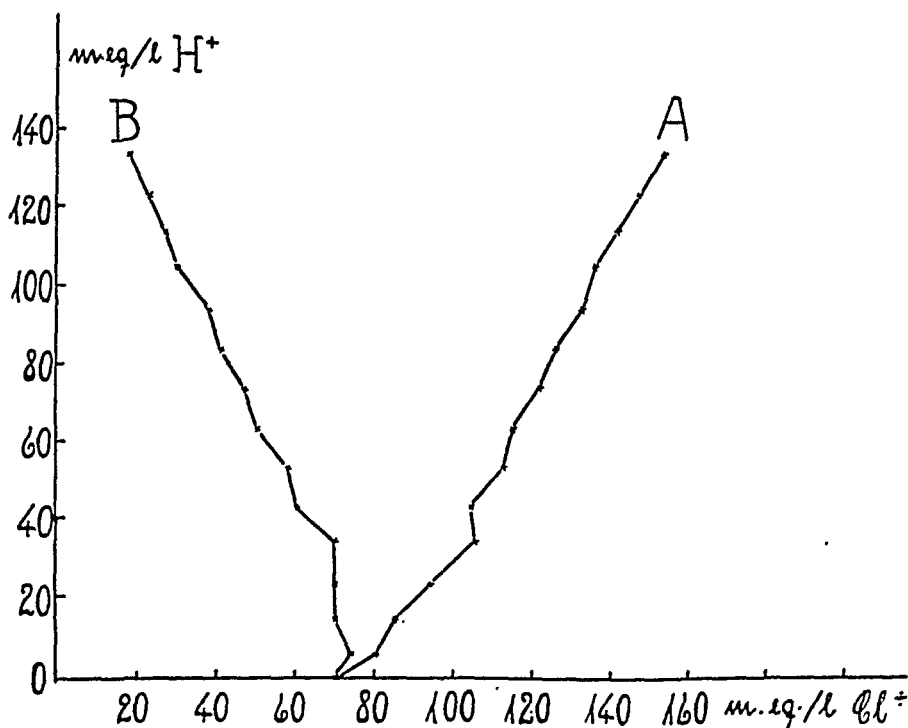


Fig. 2. Secretion after insulin stimulation. Relation between acidity and chloride.

Abscissa: Chloride. Ordinate: Acidity.
A: Total chloride. B: Neutral chloride.

The neutral chloride curve can be obtained by means of the total chloride curve, the neutral chlorides, $n, = k - m$, or $k = n + m$. By insertion of this in the equation $m = 1.73 - 1.88$, $m = 1.73 (m + n) - 133$ from which results $m = 182 - 2.87 n$. This line and the horizontal axis intersect at the point $m = 0$, $n = 77$, while the mentioned line and the vertical axis intersect at the point $m = 182$, $n = 0$, i. e. neutral chloride 0.

The idea presents itself to test the general theories of gastric secretion in respect to the equations found as outlined above. It is directly to be seen that the hypothesis of ROSEMAN of the constancy of the total chlorides and the mutual independence of the total chlorides and the acidity does not agree with the results found; on the contrary, the established curves show a close interdependence of these two factors, and that the chlorides increase simultaneously with the acidity, wherefore the hypothesis mentioned must be abandoned.

The other hypothesis is that the juice, containing hydrochloride acid, is secreted with a constant maximal acidity, and that the various acidities met with are due to dilution with a neutral or alkaline fluid. In order to test this it is necessary to clarify what the relation between the acidity, total chlorides and neutral chlorides would be according to the said hypothesis.

In the following

x = the entire secreted volume of gastric juice per time unit.

y = the volume of acid gastric juice secreted per time unit.

a = the volume of non-acid juice secreted per time unit.

m = m.eq./liter of acid chloride. (= m.eq./liter acid).

n = m.eq./liter of neutral chloride.

k = m.eq./liter of total chloride of the entire gastric juice.

p = maximal value of acid chloride in m.eq./liter (= maximum acidity).

q = maximal value of neutral chloride in m.eq./liter.

It is now assumed that during the secretory process the volume y of the fluid with constant, maximal, acidity and chloride content of p m.eq./liter is mixed with the volume a of non acid secretion with the chloride content q m.eq./liter, producing the combined volume x , with acid chloride (= acidity) m m.eq./liter, neutral chloride n m.eq./liter and total chloride k m.eq./liter.

To begin with it is assumed that the volume a of non acid, diluting fluid secreted per time unit is constant. The amount of

acid produced per time unit is $y \cdot p$ (the volume of acid juice multiplied with its content of chloride). It is presupposed that the acid juice is a pure hydrochloric acid solution. The acidity of it is then = the hydrogen ion concentration in m.eq./liter = the chloride concentration in m.eq./liter.

The actual acidity m is then = the produced amount of acid divided by the total produced volume of gastric juice, $= \frac{y \cdot p}{x}$.

$$\text{Therefore } m = \frac{y \cdot p}{x}.$$

As $x = y + a$, the equation will be

$$1) \quad m = \frac{y \cdot p}{y + a}, \text{ or } my + ma = y \cdot p, \text{ or } y = \frac{ma}{p - m}.$$

Likewise

$$n = \frac{a \cdot q}{x} = \frac{a \cdot q}{y + a}.$$

from which results $yn + an = aq$, or

$$2) \quad y = \frac{a(q - n)}{n}.$$

Expression 2) is now inserted in 1), giving

$$m = \frac{\frac{a(q - n)p}{n}}{\frac{a(q - n)}{n} + a} = \frac{a(q - n)p}{a(q - n) + an} = \frac{(q - n)p}{q} = p - \frac{p \cdot n}{q}$$

which may also be expressed:

$$n = \frac{q(p - m)}{p}.$$

Thus a theoretical equation has been established between acidity and neutral chlorides based on the assumptions mentioned. It will be noted that the expression represents a straight line. m being ordinate and n abscissa, the line intersects the horizontal axis in the point q ($m = 0$) and the vertical axis in the point p ($n = 0$).

According to this theory the equation between the acidity and the neutral chlorides must be a straight line. This is as shown also the case according to the experimental results.

In a quite similar manner the theoretical equation between total chloride and acidity is found. Using the same symbols the equations will be

$$k = \frac{yp + aq}{x} = \frac{yp + aq}{y + a}.$$

As $y = \frac{ma}{p - m}$ (1), insertion of this gives

$$\begin{aligned} k &= \frac{\frac{ma}{p - m} \cdot p + aq}{\frac{ma}{p - m} + a} = \frac{map + aq(p - m)}{ma + a(p - m)} = \frac{mp + q(p - m)}{m + (p - m)} = \\ &= \frac{mp + pq - mq}{p} = \frac{m(p - q)}{p} + q, \end{aligned}$$

from which results

$$\frac{m(p - q)}{p} = k - q; \quad m = \frac{p(k - q)}{p - q}.$$

This equation also represents a straight line.

If $m = 0$, $k = q$. If $m = k$ (all chloride being acid chloride), $m = k = p$.

The theoretical equation thus established between acidity and total chloride is now compared to the curve found experimentally. This intersects the abscissa axis in the point $m = 0$, $k = 77$. m and k are identical in the point $m = k = 182$. At acidity 0 the total chlorides are therefore $= 77$ ($=$ the neutral chlorides). At acidity 182 all chloride is present as acid chloride; this value must be the maximum acidity.

The conclusion of these computations must be that the varying relation found between acidity and chloride of the gastric juice may be explained by the assumption that a hydrochloric acid solution of a constant acidity of app. 180 m.eq./liter is mixed in the stomach with a neutral secretion having a chloride concentration of app. 75 to 80 m.eq./liter.

It is finally possible that the diluting non-acid secretion might be alkaline. This may partly be due to mucus, partly to duodenal juice (pancreatic juice). In mucus IHRE finds an alkalinity of 3.2—10.8 m.eq./liter, whereas BOLTON and GOODHEART find an alkalinity of 40 m.eq./liter.

In the duodenal juice WILHELMJ, FINEGAN and HILL find an alkalinity of 40 m.eq./liter.

Considering conditions, if the diluting secretion is alkaline, the alkalinity of the diluting juice being r m.eq./liter OH^- , we have the following equations, in analogy with page 008

$$m = \frac{yp - ar}{y + a}, \quad ym + am = yp - ar$$

$$y(p - m) = am + ar, \quad y = \frac{am + ar}{p - m}.$$

Further

$$n = \frac{aq + ar}{y + a}, \quad yn + an = aq + ar.$$

$$2) \quad y = \frac{a(q + r - n)}{n}.$$

Insertion of 2) in 1) gives:

$$\begin{aligned} m &= \frac{\frac{a(q + r - n)p}{n} - ar}{\frac{a(q + r - n)}{n} + a} = \frac{a(q + r - n)p - arn}{a(q + r - n) + an} = \\ &= \frac{pq + pr - pn - nr}{q + r}. \end{aligned}$$

From this results:

$$n = \frac{pq + pr - mq - mr}{p + r}.$$

$$\text{If } m = 0, \quad n = \frac{p(q + r)}{(p + r)}. \quad (3).$$

$$\text{If } n = 0, \quad m = p.$$

The expression represents a straight line, intersecting the vertical axis in the point p and the horizontal axis in the point

$$\frac{p(q + r)}{p + r}.$$

As the neutral chlorides are lower in patients with absolute anacidity than in anacide samples of juice from patients otherwise able to secrete acid, this may indicate that the chlorides in the latter case partly originate from neutralized acid chloride. If the average chloride value in patients with absolute anacidity (mean of twenty samples 57 m.eq./liter) be considered the proper neutral chloride, the value of q in equation 3) is 57, and according

to the results previously found $n = 77$ and $p = 182$. From this r is found to be 35 m.eq./liter OH^-

This is in accordance with the results found by BOLTON and GOODHEART and WILHELMJ, FINEGAN and HILL, concerning the chlorides in gastric mucus and duodenal juice, respectively.

In the preceding statements the neutral chloride concentration, q , has been considered constant. The possibility may also be that it varies with the acidity. In such case q in the equation

$$n = \frac{q(p-m)}{p} \text{ must be replaced with } q_1 + \alpha m \text{ or } q_1 - \alpha m$$

depending on whether the neutral chloride concentration is supposed to increase or to decrease with the acidity. α is a constant of suitable size, figured to be positive.

Considering the first possibility the equation will be

$$n = \frac{(q_1 + \alpha m)(p-m)}{p} = \frac{pq_1 + p\alpha m - mq_1 - \alpha m^2}{p}.$$

When $m = 0$, $n = q_1$.

When $n = 0$ follows $\alpha m^2 = m(pa - q_1) - pq_1 = 0$.

Determination of m through this equation gives $m = p$ and $m = -\frac{q_1}{\alpha}$. The equation represents a parabola with its axis parallel to the abscissa axis and opening against the negative direction of this axis. Only the distance between $m = 0$ and $m = p$ is of importance in this connection.

The second possibility gives an equation representing a parabola with the axis parallel to the abscissa axis and opening against the positive direction of this axis (Fig. 3).

Thus it will be noted that under the assumption that the neutral chloride concentration varies with the acidity a parabolic interdependence of chloride and acidity will result. However, it will be noted from Fig. 2 that the interdependence of chloride and acidity is very nearly rectilinear. Only the lowest acidities show a little irregularity, which — however — rather represents accidental deviations than systematical curving.

The conclusion must therefore be that there can hardly be a question of any change of the neutral chlorides following changes of the acidity, at any rate it is so small that it must be considered of no practical significance.

Summarizing, it may be stated that the varying relation found, between acidity and chloride in the gastric juice can be explained

from the supposition that a hydrochloric acid solution of a constant, maximal, acidity of app. 180 m.eq./liter is mixed in the stomach with a non acid secretion with a chloride concentration of app. 75 to 80 m.eq./liter. The chlorides of this diluting secre-

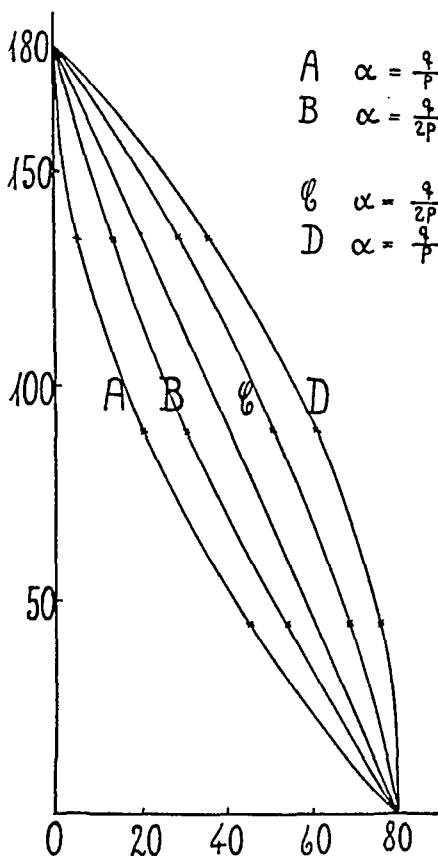


Fig. 3. Theoretical relation between acidity and neutral chloride, if the chloride concentration of the neutral secretion varies with the acidity.

A and B: Increasing acidity associated with diminished neutral chloride secretion.

C and D: Increasing acidity associated with increased neutral chloride secretion.

Straight line: Neutral chloride secretion independent of changes of acidity.
Abscissa: Chloride. Ordinate: Acidity.

tion does not vary with the acidity of the gastric juice. At the same time supposing that the diluting secretion possesses an alkalinity of app. 35 m.eq./liter OH^- it can be explained that the average chloride content is lower in juice from patients with absolute anacidity than in anacide samples of juice from persons, who otherwise through adequate stimulation are able to secrete acid juice.

Attention is drawn to the fact that the chloride concentration of the hydrochloric acid component is found to be approximately 180 m.eq./liter, *i. e.* more than the total base concentration of blood. With a total chloride concentration of gastric juice of 160 m.eq./liter, which corresponds to the total base concentration of blood, we find according to the above equations an acidity of 144 m.eq./liter and neutral chloride of 16 m.eq./liter. According to Table 1 these values represent exactly the maximum values actually observed. Should the parietal secretion consist of pure hydrochloric acid, this must consequently be of higher osmotic concentration than blood. Should the parietal secretion be isotonic with blood, it must contain a certain amount of neutral chloride. Should — according to the first possibility — the parietal secretion be of a higher osmotic concentration than blood, it is presumably very unstable in the stomach, as in no case an acidity corresponding to a solution of a higher osmotic concentration than blood has been met with in these experiments. Such hypertonic solutions may possibly by diffusion be changed into solutions isotonic with blood.

It is interesting to note that the theoretical maximum here found for the concentration of the hydrochloric acid, namely 182 m.eq./liter, is in accordance with the results obtained by IHRE from experiments on human beings using a glycol buffer solution. In this way he found an average maximum total chloride concentration of 184 m.eq./liter. Further this value is very close to that recorded by GUDIKSEN, who found a maximum acidity of 190 m.eq./liter in cats.

HOLLANDER has demonstrated a rectilinear relation between corresponding acidity and total chloride values in pouch juice from dogs, and in another work has found, by extrapolation, that the acidity of the secretion from the oxyntic cells is 170 m.eq./liter.

NORDENFELT and TEORELL have determined the relation between the acidity and the total chlorides in Ewald test meals. They have thus established the formula

$$\text{Chloride} = 0.69 \cdot \text{free acidity} + 51 = 0.75 \cdot \text{total acidity} + 40.$$

These equations also represent straight lines, yet a little different from that established in this work for the relation between acidity and total chloride. The reason for this may be that the proportion chloride — acidity is different in gastric juice and in Ewald test meals. This is highly probable, as this latter in itself must also contain chloride.

The Neutral Chlorides.

The calculations mentioned above have shown that the average chloride concentration of the neutral fluid, which is supposed to dilute the acid secretion, must be on an average 75—80 m.eq./liter. It must expressly be pointed out that it is not a question of a single, well defined secretion from a special type of glandular cells, but of a mixture of the various secretions, which may possibly dilute the product of the oxyntic cells. It may be the secretion of the peptic cells, the pyloric cells, the mucus or the mucoid cells. Finally, the saliva is of some and the duodenal juice of considerable importance.

In the literature only sparse information is found regarding the chloride content of these various secretions.

The composition of the secretion of the peptic cells is unknown. Judging from the topographical distribution of the individual cell types of the stomach, it is probable that the peptic cells are of considerable importance, and it is possibly their secretion, which constitute the essential part of the neutral fraction of the gastric juice. This assumption, however, is only hypothetical.

In the pyloric juice from dogs IVY finds a chloride concentration of 0.458—0.519 % (129—146 m.eq./liter), whereas TAKATA reports 0.53 % Cl (150 m.eq./liter).

The chloride content of mucus BOLTON and GOODHEART give as 0.36—0.50 % (101—141 m.eq./liter), while WELIN and FRISK find app. 100 m.eq./liter Cl. In dogs and cats BAXTER finds that mucus, elicited by stimulation of the sympathetic nerves or by small refractory epinephrin doses, has a chloride concentration of 0.38—0.45 % (107—127 m.eq./liter). According to IHRE the chloride concentration of mucus is 90—100 m.eq./liter (in man), but following stimulation may increase to 145 m.eq./liter.

I have myself had opportunity to examine a patient with pernicious anemia, with a complete histamine refractory anacidity. In this patient it was possible to obtain from the stomach — admixture of saliva being prevented — large amounts of a clear, extremely viscid, mucous fluid, the chloride concentration of which varied from 45 to 26 m.eq./liter. It must be supposed, that it has been pure mucus, thus having had a considerably lower chloride concentration than reported elsewhere in literature. Of course it cannot be ascertained in this particular case that it has been a question of normal gastric mucus, even if the mucus

producing surface epithelium cells according to MEULENGRACHT'S investigations are well preserved in cases of pernicious anemia. On the other hand it must be presumed difficult to collect mucus separately when experimenting with animals. BOLTON and GOODHEART have used cat stomachs under influence of atropin, presuming that atropin inhibits hydrochloric acid secretion and only permits mucus secretion, a presumption not in accordance with results reported by other authors. The same is the case with BAXTER'S experiments with stimulation of sympathetic nerves or injection of epinephrin, nor is it in this case certain, or even probable, that acid secretion and peptic cell secretion are prevented.

HAMMARSTEN reports a chloride concentration of 0.36—0.45 % (102—124 m.eq./liter) in hepatic bile and 0.08 % (23 m.eq./liter) in bladder bile.

Pancreatic juice according to STARLING has a chloride concentration of 0.28—0.30 % (79—85 m.eq./liter).

Usually a certain amount of saliva is found in the gastric juice. The chlorides of saliva is according to WELIN and FRISK app. 15 m.eq./liter. My investigations have likewise shown a chloride content in saliva of app. 15—20 m.eq./liter.

Summary.

An account is given of current theories concerning secretion of gastric juice, and of the way in which the stomach regulates the acidity of its contents.

The acidity, chloride, pepsin and rate of secretion is determined in a series of samples of gastric juice obtained from human beings, partly without stimulation, partly with stimulation by intravenous injections of insulin. Acidity and chloride show a strong parallelism, in common fasting juice as well as in secretion following stimulation by insulin. The graphic expression of the relation between acidity and chloride is very nearly rectilinear.

Calculations based on the secretory hypothesis of PAVLOV show that a rectilinear interdependence of acidity and chloride must be expected, when considering the actual acidity of the gastric juice as resulting from a mixture of an acid secretion of constant acidity with a non acid secretion. The acid component, which is supposed to be a solution of pure hydrochloric acid, is computed to have an acidity and a chloride concentration of approximately 180 m.eq./liter, whereas the chloride of the non

acid component is approximately 70—80 m.eq./liter. The computations further show that the non acid secretion probably possesses an alkalinity of approximately 35 m.eq./liter. It is not likely that the chloride concentration of the non acid component is dependent on the acidity.

The chlorides of the non acid diluting fluid probably originate from various sources.

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Demonstration of Tumorigenic Decomposition Products of 2, 3-Azotoluene.

By

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In previous investigations STRÖMBECK (1945, 1946) was able to show that the appearance of tumours in the urinary bladder after administration of 2,3-azotoluene requires contact between the vesical mucosa and the urine. This observation appears to be of considerable significance to further studies on the action of azotoluene.

Various experiments appear to justify the assumption that the tumour-producing compound is excreted with the urine — whether the substance be azotoluene or some decomposition products of azotoluene.

1. Demonstration of Aminocresol, Aminobenzoic Acid (and Toluidine) as Decomposition Products of Azotoluene.

Certain reports in the literature indicate that azo-compounds are broken down in the organism. ELSON and WARREN (1944) were able after administration of azobenzene to rats to isolate aniline from the urine; and after administration of ortho-amino-azotoluene to rabbits HASHIMOTO (1935) isolated acetylated para-toluylylene-diamine from the urine. After administration of para-dimethyl-amino-azobenzene STEVENSON et al. (1942) found amino-

phenol and para-phenylenediamine. A well-known example of these processes is the decomposition of prontosil in the organism.

From a theoretical point of view we might expect 2-3-azotoluene to break down and give rise to ortho- and meta-toluidine. Probably the amino groups of these substances may undergo acetylation. Even changes of oxidative nature are conceivable, however. Thus aniline is oxidized in the organism to para-aminophenol; and, analogously, one might imagine an oxidation of toluidine to aminocresol. On the other hand toluene may be oxidized to benzoic acid, and — in keeping with this — we might expect that aminobenzoic acid might be derived from toluidine. This assumption finds support in the fact that JAFFE and HILBERT (1888) after administration of acetylated meta-toluidine to rabbits were able to isolate acetylated meta-aminobenzoic acid from the urine. Another conversion seems possible: Besides aniline, ELSON and WARREN found also benzidine in the urine; and it is conceivable that methylbenzidine might be derived from azotoluene.

If any of these compounds are formed in the organism, after hydrolysis — with a view to the possible acetylation — the urine should contain also elements consisting of a benzene ring with a primary amino group. Such elements may be demonstrated by means of the MARSHALL reaction, *i. e.*, the primary aromatic amino group is diazotized, whereafter it can be coupled to ethyl- α -naphthylamine, giving a red colour.

Experiments showed indeed that the urine from rats treated with azotoluene gave a distinct Marshall reaction, which became more intense after hydrolysis of the urine. It is to be pointed out that azotoluene by itself gives no MARSHALL reaction, not even after hydrolysis.

According to the above, then, it would seem reasonable to expect both acids and bases as decomposition products of azotoluene. But, as possibly occurring acetylated products appear to be of minor interest in this connection, the isolation experiments were planned in principle as follows:

The urine was hydrolyzed by boiling with hydrochloric acid (100 cc. hydrochloric acid added per liter of urine), and then extracted with ether. After neutralization with sodium hydroxide, and alkalization with sodium bicarbonate, the urine was again extracted with ether. Aminobenzoic acid as well as toluidine and aminocresol give hydrochlorides that are insoluble in ether. Aeration with gaseous HCl of the extracts from acid as well as from alkaline urine gave precipitation.

Table 1.

Marshall's Reaction Performed on Toluidine, Aminobenzoic Acid, Aminocresol and a Urinary Preparation in Varying Milieu.

The extinction values recorded were read with ZEISS' PULFRICH Photometer, Filter S 53 (see the text).

	HCl NaCl	Direct	Neutraliza- tion primary phosphate	Neutraliza- tion pH 7	Non-diazo- tized NaOH
Meta-toluidine	0.02	0.15	0.53	0.60	0.0
Ortho-toluidine	0.01	0.04	0.21	0.79	0.0
Meta-aminobenzoic acid ..	0.64	0.63	0.62	0.60	0.0
Ortho-aminobenzoic acid..	0.09	0.21	0.40	0.46	0.0
Aminocresol	0.04	0.10	0.10	0.13	0.15
Urinary preparation	0.36	0.43	0.36	0.32	0.02

The precipitate from the alkaline extract was dissolved in dilute HCl, alkalinized again with sodium bicarbonate, whereafter this alkaline solution was extracted with ether. The ether was evaporated, and the remnant was submitted to sublimation, yielding a white preparation. On elementary analysis this substance gave the values 68.9 % for carbon and 7.21 % for hydrogen. This means that aminocresol had been isolated (its theoretical values are respectively 68.3 and 7.37). (Studies as to whether the isolated aminocresol preparation contains the ortho or the meta form have not been carried out yet).

As mentioned before, also the extract from the acid urine contains an ether-insoluble hydrochloride. This was dissolved in bicarbonate and the alkaline solution was acidified slightly with hydrochloric acid, giving a precipitate. This was submitted to several crystallization and sublimation experiments, all of which turned out negative (only small amounts of the substance were available). Thus, it has not been possible so far to produce any pure preparation of this kind. Still, as shown in Table 1, with various modifications of the MARSHALL reaction, this preparation behaves like meta-aminobenzoic acid. Meta-aminobenzoic acid and the urinary preparation give the same extinction values (ZEISS' PULFRICH photometer, filter S 53) in all the cases where a diazotization preceded the addition of the reagent, while other substances, included for comparison, give characteristic variations with a marked fall in the extinction values in acid reaction milieu.

(Usually MARSHALL's reaction is carried out with diazotization in 1 n hydrochloric acid. Addition of the reagent to this solution corresponds to the designation "direct" in Table 1. The other column-heads imply either addition of 5 n hydrochloric acid and concentrated NaCl solution or neutralization with sodium hydroxide and then addition of primary phosphate or phosphate buffer with pH 7. Substances containing a hydroxyl group as well as an amino group give also without diazotization in alkaline solution a colour reaction with the reagent.

This is recorded in the last column of Table 1. A more detailed account of the different variations of the reaction will be given in a subsequent paper.)

So we suppose that meta-aminobenzoic acid is a decomposition product of 2,3-azotoluene.

It seems justified to assume, however, that the formation of aminocresol and aminobenzoic acid has been preceded by a breakdown of the azotoluene molecule in ortho- and meta-toluidine.

None of the extracts obtained in various ways in the course of these processes contained any substance that could be taken to be unchanged azotoluene.

2. Administration of Ortho and Meta Toluidine to Rats Resulting in Tumours in the Urinary Bladder.

Aminobenzoic acid and aminocresol may reasonably be looked upon as detoxication products of toluidine. It seemed obvious therefore among the demonstrated decomposition products to pick out the toluidines for experiments aimed at the production of tumours in the bladder.

Rats were given a basic diet of rice flour with addition of a slice of carrot about every other day, and toluidine was added to the rice flour in such a concentration as might reasonably result from a total break-down of the amount of azotoluene given to rats in previous experiments (STRÖMBECK 1945, 1946). Soon after, this dose had to be lowered because of its strong general effect on the animals. In the latter part of the experiment the toluidine supply to the animals corresponded to about 7.5—12 mg. per day to each animal, while in the first part of the experiment it had been twice as high.

The experiment comprised two series: one series of 10 rats given ortho-toluidine, the other comprising 9 rats which received meta-toluidine. On an average, the ortho-toluidine animals lived 91 days after the commencement of the experiment, and 3 of them showed changes in the mucous membrane of the bladder with metaplasia and early epithelial proliferation. The average life-time of the meta-toluidine animals was 94 days, and 6 of these showed similar changes as just recorded for the ortho-toluidine animals

In the meta-toluidine series the frequency of the changes is

Table 2.

Comparison Between the Occurrence of Tumours and the Probable Decomposition Products of the Azo-compounds Examined — According to the Literature.

Compound administered (by mouth to rats)	Changes in		Probable decomposition products of the compounds given
	Liver	Bladder	
p-aminoazobenzene	neg.	neg.	aniline + p-phenylenediamine
p-dimethylaminoazobenzene (butter yellow)	pos.	neg.	aniline + p-dimethylphenylenediamine
(p-dimethylphenylenediamine)	neg.	—	(p-dimethylphenylenediamine)
2,3-azotoluene	neg.	pos.	o-toluidine + m-toluidine
4'-amino-2,3'-azotoluene (o-amino-azotoluene)	pos.	pos.	o-toluidine + p-toluylenediamine (methyl group in m-position)
4'-acetylamino-2,3'-azotoluene	pos.	pos.	o-toluidine + acet-p-toluylenediamine (methyl group in m-position)
4'-diacetylamino-2,3'-azotoluene	pos.	pos.	o-toluidine + diacet-p-toluylenediamine (methyl group in m-position)
4'-amino-2,2'-azotoluene	neg.	—	o-toluidine + p-toluylenediamine (methyl group in o-position)
4'-oxy-2,3'-azotoluene	neg.	pos.	o-toluidine + p-oxy-m-toluidine
4'-acetoxy-2,3'-azotoluene	neg.	—	o-toluidine + p-acetoxy-m-toluidine

higher than that previously found by STRÖMBECK in animals on the same diet with administration of azotoluene. The changes are not pronounced — but that was hardly to be expected after such a short experimental period — and in principle they appeared to be of the very same kind as found in the previous series of animals treated with azotoluene.

3. Is the Tumorigenic Effect of the Azo-compounds Attributable to a Few Common Decomposition Products?

Lately, great interest has been taken in the azo-compounds and, above all, Japanese investigators (list of literature given by EULER and SKARZYNSKI 1942) have in various ways altered the molecule in order to get a further insight into the connection between the constitution of the substance and its cancerogenic action. In Table 2, according to the literature available to us, we have entered the hitherto examined azo-compounds, recording their possible tumorigenic effect in the liver and bladder, besides

the decomposition products which primarily should be formed on dissociation of the azo-binding — according to the findings presented in the preceding section.

We thus found, above all, that all the compounds which may cause tumours in the bladder can give rise to ortho-toluidine and meta-toluidine or para-substituted derivatives of meta-toluidine. In view of our findings — that meta-toluidine as well as ortho-toluidine may produce tumour-like changes in the mucosa of the bladder — it seems justified to assume that the cancerogenic action of the azo-compounds in the bladder is due to generation of toluidines. In those cases where ortho-toluidine is formed together with para-substituted meta-toluidine, of course, the effect may be due to the appearance of the ortho-toluidine. Whether the para-substituted meta-toluidine has preserved its tumorigenic capacity cannot be settled yet.

4. In the Urine from Patients with Tumours of the Bladder Compounds may be isolated in the same manner as the Decomposition Products of Azotoluene containing a Primary Amino Group.

In itself it seems to be of considerable interest if the tumorigenic effect of the azo-compounds in the bladder can be referred to the simple decomposition products: toluidines. But, in addition, this gives rise to the problem, whether similar simple molecules might not be formed from some other material of origin — as, for instance, in the human organism.

In connection with certain methodological studies we have found that urine from normal persons contains very small amounts of substances giving the MARSHALL reaction. In some preliminary experiments we have looked into the occurrence of such compounds in the urine from patients with vesical tumours. The urine was hydrolyzed with hydrochloric acid and neutralized. After addition of sodium bicarbonate, one portion of the urine was extracted with ether, and another portion was submitted to steam distillation. Both procedures should yield aromatic amines in the extract, respectively in the distillate, if such bodies are present in the urine.

Indeed, this proved to be the case. The distillate was alkalinized and extracted with ether; and after evaporation of the ether, a remnant of about 10 mg. per liter of urine was obtained. Even such a small amount as 0.2 mg. of this remnant gave a distinct MARSHALL reaction, which was stronger at pH 7 than in acid solution. The direct ether extract

of the urine was evaporated, and 1 mg. of the remnant gave a MARSHALL reaction of about the same intensity as that obtained with 0.2 mg. of the rest from the distillation. The total amount of the substance obtained by direct ether extraction, however, was considerably larger. It is to be pointed out that here a distinct reaction was obtained even without diazotization. So it appears as if the urine from these vesical tumour patients contains not altogether inconsiderable amounts of primary aromatic amines, some of which might even be present in oxidized form.

Further experiments indicate that it is possible by distillation of small amounts of urine to obtain destillates that can be employed for reproducible quantitative determinations of these aromatic amines.

Collection of larger amounts of urine is now going on in order, if possible, to obtain sufficient material for identification of these compounds. Further, comparative studies are planned on the excretion of these substances in various tumour patients and normal persons.

Summary.

1. Rats given 2,3-azotoluene excrete aminocresol and aminobenzoic acid with the urine and it is assumed that ortho- and meta-toluidine are the primary decomposition products.

2. Administration of ortho- and meta-toluidine to rats results in tumours in the urinary bladder.

3. It is pointed out that all the hitherto examined tumorigenic azo-compounds according to our findings might give rise to ortho- or meta-toluidine and we assume that the tumorigenic action of the azo compounds in the bladder is due to generation of toluidines.

4. In the urine from patients with tumours of the bladder, compounds, containing a primary amino group, may be isolated in the same manner as the decomposition products of azotoluene.

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Methods for Quantitative Determination in Urine of Aminobenzoic Acid, Aminocresol and Meta-toluidine.

By

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In a preceding paper (EKMAN and STRÖMBECK 1947) it was shown that 2,3-azotoluene is broken down in the organism, and that among the decomposition products, aminocresol, aminobenzoic acid and ortho- and meta-toluidine — at any rate the two toluidines — have a tumorigenic effect. Previously STRÖMBECK (1945 and 1946) had been able to show that azotoluene gives rise to tumours in rats on a diet consisting chiefly of rice flour, whereas an adequate diet (HAMMARSTEN's (1937) diet No. 211) with the same azotoluene supply gave no tumours. This protective effect may be due either to some influence of the diet upon the mucous membrane of the bladder or to the conversion of azotoluene in the organism.

If it might be possible to determine the decomposition products from azotoluene on the various diets, and if the excretion of some of these products with the urine might be affected by the diets, we might assume that the protective effect would be due to interference with the metabolism of azotoluene. In order to make it possible to carry out studies concerning this question, the following methods for quantitative determination were worked out.

1. Determination of Meta-aminobenzoic Acid.

In attempts to estimate the decomposition products of 2,3-azotoluene we have to reckon with their simultaneous occurrence in the urine, and the methodological work must be aimed at the

elaboration of methods allowing determination of each product separately. All the decomposition products contain a primary amino group, and thus their presence can be established by means of MARSHALL's reaction.

SIMESSEN (1939) has given a modified technique for determination of a sulfapreparation with this reaction. For determination of the acetylated forms she employed hydrolysis by boiling with 30% sodium hydroxide. In preliminary experiments on the employment of this technique for determination of toluidine, the values obtained were very low even for high concentrations. As a matter of fact, these findings might be taken to indicate that the hydrolysis with sodium hydroxide destroyed the toluidine. But, then it was found that low values were obtained also without boiling and on immediate neutralization of the sodium hydroxide with hydrochloric acid. It then seemed conceivable that the inhibition of the colour reaction of toluidine might be due to a high sodium chloride concentration and acid reaction. Indeed, a very strong effect could also be obtained by hydrolysis in acid solution and, after this, addition of concentrated sodium chloride solution and additional hydrochloric acid. In this way the colour reaction could be suppressed entirely even with relatively high toluidine concentrations. However, the same procedure gave normal values for meta-aminobenzoic acid. After this principle, then, the following technique was worked out:

The 24-hour urine of the rat is diluted to 50 cc. and filtered. (This dilution is suitable when the rats daily receive about 30 mg. azotoluene or 10 mg. meta-toluidine.) Of this urine, 0.5 cc. is placed in a test tube together with 2 cc. 1 n HCl. For comparison, in another test tube the urine is replaced with 0.5 cc. of water. The test tubes are equipped with a simple "cooler", consisting in a glass ball which covers the mouth of the tube and is kept in place by means of a stem dipping down in the tube. The test tubes are left standing on a boiling water-bath for 30 minutes. After cooling, 2 drops of 1 % sodium nitrite solution are added and, after about 2 min., 1 cc. of 2 % urea solution. (In order to obtain correct values, it is very important that the sodium nitrite solution does not fasten on the wall of the tube.) The tube is left standing for at least 30 min. Then 1 cc. 5 n HCl and 2 cc. 25 % NaCl are added. After shaking, 1 cc. 0.3 % ethyl- α -naphthylamin-hydrochloride solution in 1 n HCl is added and 5 cc. alcohol. Shaking. After 2 hours, reading of the results in a Zeiss' Pulfrich photometer with filter S 53 and with 10 mm. cuvettes.

Each time new solution is prepared, the method ought to be tried out with a solution containing 5 mg. meta-aminobenzoic acid in 100 cc. 0.1 n HCl.

Comments of the Method.

Table 1 gives the outcome of determinations with this technique of the same amounts of ortho- and meta-aminobenzoic acid, of the double of ortho- and meta-toluidine and aminocresol. Thus the toluidine and aminocresol are found not to interfere with the determination of meta-aminobenzoic acid. Ortho-aminobenzoic acid gives an extinction value which amounts only to about 15 % of the value for meta-aminobenzoic acid.

From Table 2 it will be noticed that, with the technique here employed, the urine from animals, which have received 2,3-azotoluene or meta-toluidine, on hydrolysis gives increased extinction values, and that the hydrolysis appears to be concluded after 30 min. The values indicate that aminobenzoic acid is excreted mostly in acetylated form.

Table 1.

Comparison between the Extinction Values for some Substances which conceivably may interfere with the Determination of Meta-aminobenzoic Acid.

	mg.	E
Ortho-aminobenzoic acid	0.025	0.09
Meta-aminobenzoic acid	,	0.64
Meta-toluidine	0.05	0.02
Ortho-toluidine	,	0.01
Aminocresol	,	0.04

Table 2.

Influence of varying Length of the Hydrolyzing Time. Determination of the Meta-aminobenzoic Acid Content of Urines from Animals given Meta-toluidine or 2,3-azotoluene.

Boiling time in minutes	0	15	30	50
Treatment with meta-toluidine:				
Urine I	0.09	0.47	0.55	0.56
Urine II	0.13	0.36	0.49	0.49
Treatment with azotoluene:				
Urine I	0.17	0.42	0.54	0.57
Urine II	0.11	0.35	0.46	0.42

Table 3.

Extinction Values obtained with varying Reading Time. Determination of Meta-aminobenzoic Acid in Urine from Rats given Meta-toluidine or 2,3-azotoluene.

Time after addition of reagent in min.	20	50	80	100	160
Treatment with meta-toluidine	0.27	0.30	0.28	0.32	0.33
Treatment with azotoluene . .	0.26	0.35	0.35	0.37	0.38

Table 4.

Extinction Values for 0.025 mg. Meta-aminobenzoic Acid in Aqueous Solution or added to various Urines from Animals on Different Diets.

	Protective diet			B-free diet	
Treatment with azotoluene . .	0.62	0.60	0.60	0.61	0.60
Treatment with meta-toluidine	0.63	0.60	0.62	0.63	0.61
Meta-aminobenzoic acid in aqueous solution		0.62	0.60	0.62	

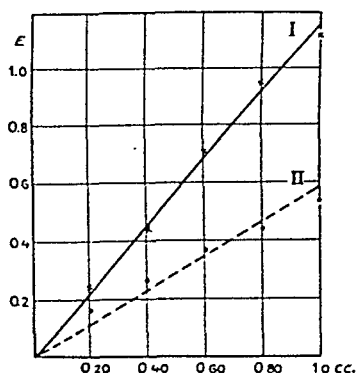


Fig. 1. Extinction values for varying amounts of the same urine from rats given 2,3-azotoluene (I) and from rats given meta-toluidine (II).

According to Table 3, when the extinction values are read after 100 min. they are constant. According to Table 4, the same reading values are obtained for meta-aminobenzoic acid in aqueous solution as for the same amount of meta-aminobenzoic acid added to urines from animals given azotoluene or meta-toluidine and kept on a protective diet as well as on a tumorigenic diet. (The urines from the animals on protective diet contain relatively large amounts of ascorbic acid.)

That there is a direct proportionality between the extinction value and the concentration is evident from Fig. 1, in which the extinction value for dilutions of urine from animals given azotoluene or meta-toluidine proved to lie on a straight line.

2. Determination of Aminocresol.

In attempts to differentiate the performance of MARSHALL'S reaction in such a way that only aminocresol — but not aminobenzoic acid or toluidine — gives the reaction, it was found that aminocresol gives a colour reaction in alkaline solution also without preceding diazotization. While the usual colour reaction with MARSHALL'S reagent gives a reddish-violet colour, the colour here observed is bluish-violet. Still, both give an extinction maximum in the Pulfrich apparatus with filter S 53. As far as I have been able to find out, the reaction has not been employed previously for quantitative or qualitative determinations. Aminocresol contains a hydroxyl, a methyl and an amino group, linked to a benzene ring. As neither toluidine (with one amino group and one methyl group) nor aminobenzoic acid (with one amino group and one carboxyl group) give this reaction, it seemed rather likely to be due to the amino and hydroxyl groups. Indeed this proved to be the case as no reaction was obtained with phenol (one hydroxyl group) or aniline (one amino group) whereas para-aminophenol (with both an amino group and a hydroxyl group) gives the reaction.

Even without addition of the reagent, a reddish colour is obtained from aminocresol with alkali in the concentration here employed. This colour is considerably weaker, however, besides being unstable. The disturbing influence of this dye production can be eliminated by using the same urine with addition of alkali, but no reagent, for comparison.

For quantitative determination of aminocresol the following technique was elaborated:

The 24-hour urine is diluted to 50 cc. and filtered. Of this filtrate, 1 cc. is placed in each of two test tubes, and then 2 cc. 1 n hydrochloric acid. "Cooler" as described in Section 1, boiling on water bath for 30 min. After cooling, 2 cc. 2 n sodium hydroxide is added to either tube. To one tube is now added 1 cc. 1 n hydrochloric acid, to the other 1 cc. 0.3 % ethyl- α -naphthylamin-hydrochloride solution in 1 n hydrochloric acid. Further 5 cc. alcohol is added to either tube. After 60 min., the values are read with filter S 53 and 10 mm. cuvettes in Zeiss' Pulfrich photometer, one cuvette containing the solution to which the reagent was added, the other cuvette containing the other solution for comparison.

Each time a new solution is prepared, the method ought to be tried out with a solution of aminocresol. In my experiments I have used 10

mg. aminocresol in 100 cc. 0.1 n HCl. It is to be pointed out that my preparation probably is a mixture of ortho-aminocresol and meta-aminocresol.

Comments of the Method.

As mentioned above, aminobenzoic acid and toluidine do not give this reaction at all. *Table 5* shows that a hydrolyzing time of 30 min. is sufficient and that aminocresol plainly is excreted entirely in acetylated form. After 60 min., constant reading values were obtained, and they may keep unchanged even after 24 hours.

Table 5.

Varying Hydrolyzing Time in Determination of the Aminocresol Content of Urine from Rats given Meta-toluidine or 2,3-azotoluene.

Boiling time in min.	0	15	30	60
Treatment with meta-toluidine	0.0	0.04	0.055	0.06
Treatment with azotoluene . .	0.0	0.09	0.18	0.19

Table 6.

Extinction Values for varying Amounts of Aminocresol in Aqueous Solution or added to Urines from Rats on different Diets.

Aminocresol in mg.	0.05	0.10	0.15	0.20	0.30
Protective diet	0.08	0.19	0.29	0.42	0.65
B-free diet	0.06	0.18	0.33	0.44	0.68
Aqueous solution	0.07	0.16	0.27		

According to *Table 6* the extinction values for increasing amounts of aminocresol remain the same, no matter whether aminocresol is added to urine with low or high ascorbic acid content (from animals kept on B-free diet or on protective diet) or dissolved in water. Calculation of the ratio between the concentration values and extinction values recorded in *Table 6* shows a direct proportionality.

3. Determination of Meta-toluidine.

In the preceding it has been shown that by addition of strong hydrochloric acid and concentrated sodium chloride solution it is possible to suppress the reaction between toluidine and

Table 7.

Extinction Values for 2,3-azotoluene Decomposition Products in various Modifications of the Marshall Reaction (see the text).

mg.		HCl NaCl	Direct	Neutr. Prim. Ph.	Neutr. pH 7	No diazot. NaOH
0.05	Meta-toluidine	0.02	0.15	0.53	0.60	0.0
0.05	Ortho-toluidine	0.01	0.04	0.21	0.79	0.0
0.025	Meta-aminobenzoic acid .	0.64	0.63	0.62	0.60	0.0
0.025	Ortho-aminobenzoic acid .	0.09	0.21	0.40	0.46	0.0
0.10	Aminocresol	0.04	0.10	0.10	0.13	0.15

MARSHALL's reagent. But, if the reaction is performed with the technique employed for determination of a sulfapreparation (working in about 1 N HCl) toluidine gives some colour. It therefore seems conceivable that by further shifting of pH of the reaction solutions in alkaline direction a stronger colour reaction (dye formation) between toluidine and MARSHALL's reagent might be obtained. For this purpose experiments were carried out, in which the hydrochloric acid employed prior to the hydrolysis was neutralized and then the pH was stabilized through addition of 1,5-molar primary phosphate buffer or 1,5-molar solution of primary and secondary phosphate buffer with $\text{pH} = 7$. Finally, the test was performed in alkaline solution. As animals receiving azotoluene may be expected to excrete with the urine toluidine as well as aminobenzoic acid and aminocresol — in their ortho- and metaforms — the reaction was carried out with these substances after the principle outlined above.

From Table 7 it is evident that the highest extinction values for meta- and ortho-toluidine are obtained in neutralized solutions and on addition of a buffer with $\text{pH} = 7$. The values obtained in tests carried out on alkaline solution are not recorded, as such tests give rise to the appearance of particularly unstable dyes, which are destroyed very quickly.

Table 7 shows, however, that within the range of pH suitable for determination of meta-toluidine also small amounts of ortho- and meta-benzoic acid give an equally strong reaction. Finally, aminocresol gives reading values that may reasonably be left out of consideration. A method for determination of meta-aminobenzoic acid has been given above. But, as ortho- as well as meta-

Table 8.

Influence of Addition of Ascorbic Acid and Aminocresol on the Determination of Meta-toluidine at pH 7.

Addition of ascorbic acid in mg.	E	Addition of aminocresol in mg.	E
—	0.24	—	0.95
0.05	0.22	0.005	1.00
0.10	0.16	0.01	0.92
0.50	0.05	0.05	0.75

benzoic acid give this reaction within the range of pH suitable for toluidine determination, no correct values for toluidine may be obtained through combination of both methods. It is of interest for our investigations, however, to study the excretion of the tumorigenic compound toluidine on protective diet and on deficiency diet. So the studies were continued for the purpose of ascertaining the possibilities of toluidine determination on urine from animals given meta-toluidine, and in whose urine we thus may expect an excretion of unchanged toluidine, meta-aminobenzoic acid and aminocresol.

The extinction values at pH 7 will here depend upon the amounts of meta-toluidine and meta-aminobenzoic acid in the urine. Through simultaneous determinations of meta-aminobenzoic acid alone with the technique given above it should be possible to make a correction of the values obtained. For, from Table 7 it is evident that meta-aminobenzoic acid gives practically the same extinction values, no matter which method is employed. Experiments carried out with a view to this point, however, indicated that at pH 7 a certain amount of meta-aminobenzoic acid or meta-toluidine in the urine gives extinction values that are only one-half or one-third as large as the values obtained in an aqueous solution.

It seemed rather likely that reducing compounds in the urine might bring about this effect, and Table 8 shows, indeed, that this is the case. Small amounts of ascorbic acid and aminocresol give lowered extinction values for meta-toluidine solutions. (In neutral and slightly alkaline solutions, aminocresol is oxidized and might thus act as a reducing agent.) Experiments were thus carried out on elimination of the reducing compounds by addition

of a suitable oxidizing substance which — for instance, by its own colour — indicates when an excess of this substance is reached. Potassium permanganate, however, proved unserviceable for this purpose.

Taking for granted, however, that in urine too there is a direct proportionality between the extinction and concentration of meta-toluidine — and as will be noticed from *Fig. 2*, this is the case — another way seemed to be open — a way that might be presented most clearly, I think, by means of an example.

The urine was diluted to 50 cc., and all the determinations were carried out on 0.5 cc. of this dilution.

Employment of the technique for determination of meta-aminobenzoic acid (acid reaction) gave an extinction value of 0.50, *i. e.*, a value corresponding to the meta-aminobenzoic acid content of the urine.

On determination at pH 7, an extinction value of 0.40 was obtained, *i. e.*, a value brought about by meta-toluidine as well as meta-aminobenzoic acid.

As mentioned before, with acid reaction the extinction value for meta-aminobenzoic acid is the same in urine as in aqueous solution. A known amount of meta-aminobenzoic acid in aqueous solution was determined, and after the meta-aminobenzoic acid method it gave the value of 0.60. The same amount, added to the specimen of urine, increased its extinction value from 0.40 at pH 7 to 0.78, that is to the added amount of meta-aminobenzoic acid we have the corresponding value of $0.78 - 0.40 = 0.38$; and 0.38 makes 63.3 % of the value for the same amount of the substance in aqueous solution.

So the value of 0.50 obtained with the aminobenzoic acid determination technique for the original urine may be reduced in the same manner, that is to 0.32. Thus 0.32 is the extinction value corresponding to the meta-aminobenzoic acid at pH 7. The extinction value for the urine at pH 7 was 0.40. Diminution of 0.40 by 0.32 gives the value 0.08, which then constitutes that part of the extinction value for the urine at pH 7 that is due to meta-toluidine alone.

Now, a known amount of meta-toluidine was added to the urine, namely: 0.05 mg. By this, the extinction value at pH 7 was increased from 0.38 to 0.99. Thus, 0.05 mg. corresponds to an extinction value of 0.61; an extinction value of 1.0 corresponds to 0.082 mg.; and thus the extinction value of 0.08 for meta-toluidine in the urine corresponds to 0.00656 mg. meta-toluidine in the 0.5 cc. of diluted urine used for the tests, making the 24-hour output equal to 0.656 mg.

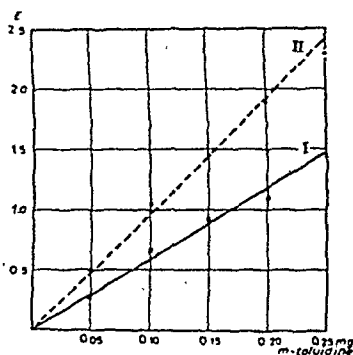


Fig. 2. Extinction values for varying amounts of meta-toluidine added to urine (I) or in aqueous solution (II).

Here then we are dealing with two calibrations: 1) one with a view to correction of the meta-aminobenzoic acid value in a fluid with acid reaction to the meta-aminobenzoic acid value at pH 7; and 2) another that involves the calculation of the amount of meta-toluidine (in mg.) that corresponds to the corrected extinction value at pH 7 in the urine under analysis. Thus meta-aminobenzoic acid as well as meta-toluidine have been added to urine samples.

It seems conceivable, however, that both substances are influenced in the same degree by the reducing compounds in the urine and that it might be sufficient therefore to add either compound to the urine. The following experiment was performed:

To 8 specimens of different urines, meta-toluidine and meta-aminobenzoic acid were added. The proportion between the extinction value for either substance in aqueous solution and in these urines at pH 7 was determined, and it was found that the value for the aqueous solution of meta-aminobenzoic acid was reduced to an average value of 64.4 %, for meta-toluidine to an average of 68.4 %. This agreement must be looked upon as sufficiently good to justify the calibration of merely one of these substances. (The percentages differed for the different urines, though with mutual agreement between the percentages for meta-toluidine and meta-aminobenzoic acid.)

Naturally, a calculation of meta-toluidine in this way — by means of the values obtained in 3 reactions with three colorimetric readings — is bound to give a considerable percental error. Still, if it is a question of demonstrating very great variations in the meta-toluidine output, it might afford some information.

For quantitative determination of meta-toluidine after the principle given above the following technique is given.

The urine is diluted to 50 cc. and filtered. From the filtrate 0.5 cc. urine is transferred to each of two test tubes, and 2 cc. *n* HCl is added to either tube. Further, 0.5 cc. water is added to one tube, 0.5 cc. meta-toluidine solution (10 mg. dissolved in 100 cc. 0.1 *n* HCl) to the other tube. Then both tubes, provided with a "cooler" are placed on boiling water bath for 30 min. After cooling, 2 drops of a 1 % sodium nitrite solution are added and, after about 2 min. 1 cc. 2 % urea solution. After shaking, the tubes are left standing at least for 30 min. Then, to both tubes are added 1 cc. 2 *n* NaOH and 2 cc. 1.5-molar phosphate buffer with pH = 7. After shaking, 1 cc. of a 0.3 % ethyl- α -naphthylamine-hydrochloride solution in 1 *n* HCl, together with 5 cc. alcohol. After thorough mixing, both tubes are read — with employment of filter S 53 and 10 mm. cuvettes — against the contents of another tube treated as above, but containing 0.5 cc. water instead of urine. The tube without any addition of toluidine gives the value for the urine, and the difference between the extinction values for the two tubes gives the extinction value for the added amount of meta-toluidine (0.05 mg.).

Every time a new solution is prepared, it is necessary to determine the extinction value for 0.5 cc. of the meta-toluidine solution without additional urine (the urine is replaced by 0.5 cc. water) in order to be

able to estimate the initial value for the calculation of the reduction percentage on addition of the toluidine solution to the urine.

The meta-aminobenzoic acid output is determined with the technique given above (p. 00).

The calculation will be evident from the example given in the description of the principle. Here, however, it must be kept in mind that for the calculation of the part of the aminobenzoic acid in the extinction value at pH 7 we employ the extinction value for the added amount of meta-toluidine for calculation of the reduction percentage.

Comments of the Method.

As mentioned before, from *Fig. 2* it is evident that there is a direct proportionality between the meta-toluidine concentration and the extinction value in urine too. As will be noticed from *Table 9* the length of the hydrolyzing time is sufficient, and *Table 10* shows that after 30 min. the extinction values keep constant.

Table 9.

Influence of Variations in the Length of the Hydrolysing time on the Extinction Values.

Boiling time in min.	0	20	30	40
Urine I	0.15	0.74	0.79	0.79
Urine II	0.44	0.95	1.08	1.08

Table 10.

Extinction Values in varying Reading Times.

Time after addition of reagent in min.	30	60
Urine I	0.14	0.14
Urine II	0.21	0.20
Meta-toluidine solution	0.64	0.68

It is to be emphasized that pH is corrected to 7 before the addition of the reagent, and that pH again is shifted to about 3.9 by the solution of the reagent (1 n HCl). It therefore seems suitable from the start to adjust pH to this value and use a reagent solution which likewise is adjusted to pH = 3.9. Experiments showed, however, that such a procedure gave entirely different, not serviceable, reactions.

In determination of primary aromatic amines, also a reagent other than ethyl- α -naphthylamine has found employment. WESTFALL (1944)

has used a modification of the BÖNIGER (1894) reaction, making use of the reaction with sodium 1,2-naphthoquinone-4-sulfonate for determination of 2-aminofluorene. The different forms of toluidine and aminobenzoic acid as well as aminocresol were found, however, to give about the same intensity of colour with this reagent even when the reaction milieu was varied in several ways. So it did not seem possible by means of this reagent to work out any simpler technique for determination of meta-toluidine than the one given here.

Summary.

Methods have been worked out allowing the simultaneous estimation of meta-aminobenzoic acid and aminocresol in urine from rats (receiving 2,3-azotoluene). The occurrence of other decomposition products, meta- and ortho-toluidine, does not interfere, but it has not been possible to determine these substances when they all are found together.

Rats given meta-toluidine will excrete meta-toluidine, meta-aminobenzoic acid and aminocresol. In this case a method has been elaborated for the calculation of each product separately.

The methods for toluidine and aminobenzoic acid are based on the employing of MARSHALL's reaction (between diazotized primary amines and ethyl- α -naphthylamin) at various pH and sodium chloride concentration.

The method for aminocresol is based on the observation that benzene compounds containing an amino and a hydroxyl group give a colour reaction with MARSHALL's reagent in alkaline solution also without preceding diazotization.

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Excretion of Decomposition Products of 2,3-Azotoluene on Various Diets.

By

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Previously STRÖMBECK (1945, 1946) has shown that administration of 2,3-azotoluene did not give rise to tumours of the bladder in rats on a laboratory diet of the type usually employed in Europe (diet No. 211, given by HAMMARSTEN, 1937). In the following, this diet is designated as *protective diet*.

On employment of a diet consisting of rice flour with addition of a small slice of carrot every other day (here designated as *rice diet*) STRÖMBECK was able to reproduce the results reported by Japanese investigators. Like OTSUKA and NAGAO (1936), he obtained certain changes in the mucous membrane of the urinary bladder. This was in keeping with the unsuccessful attempt of other European investigators (HEEP 1936; FISCHER-WASELS 1937) to obtain tumours of the liver on a European laboratory diet after administration of ortho-amino-azotoluene or para-dimethylamino-azobenzene (butter yellow), which, according to YOSHIDA et al. (1935) should be practicable.

It might be assumed that if the excretion of the decomposition products of 2,3-azotoluene (toluidine, aminobenzoic acid and aminocresol — EKMAN and STRÖMBECK 1947) is affected by the diet, the tumour protective effect would be due to interference with the metabolism of azotoluene

1. Variations in the Excretion of an Azotoluene Decomposition Product observed on changing from Protective Diet to Rice Diet.

Preliminary experiments were aimed at the excretion of primary aromatic amines in the urine from rats given azotoluene in the diet in such an amount that the daily intake of this substance was 30—45 mg. Here the same technique was employed as given

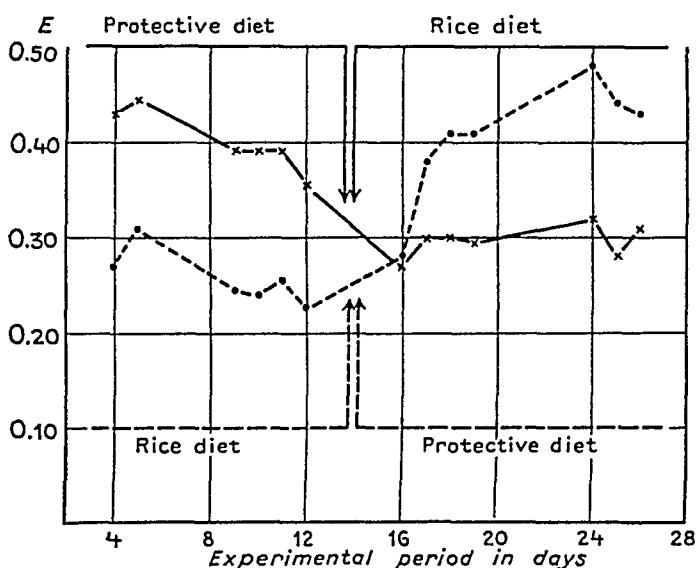


Fig. 1. Excretion of substances containing a primary amino group in rats given 2,3-azotoluene in their food (30—45 mg. per day). Extinction values determined after SIMESSEN's (1939) method for determination of sulfanilamide. Each curve corresponds to the average values for 8 animals.

by SIMESSEN (1939) for determination of sulfa-preparations. The modification comprising hydrolysis of the urine with sodium hydroxide was used. As mentioned in a preceding paper (EKMAN 1947 a), it is chiefly meta-aminobenzoic acid that is determined. As this was not known at the performance of these experiments, however, the values given in Fig. 1 are not absolute, but merely extinction values. Still, from Fig. 1 it is plainly evident that in animals on the protective diet the excretion lies at a considerably higher level than in the rice diet. Each experimental group com-

prises 8 rats. The output on protective diet is nearly twice as large as the output on the rice diet.

Thus these experiments illustrate clearly the influence of the protective diet on the excretion products. Besides rice flour, the protective diet contains also peanut oil, cod liver oil, yeast, wheat sprouts and a salt mixture.

As to liver tumours, KENSLER et al. (1941) have shown that the development of hepatomas after administration of para-dimethylamino-azobenzene is inhibited by the combination riboflavine + casein. According to the view presented in a preceding paper (EKMAN and STRÖMBECK 1947), also this compound is broken down in the organism, and the protective effect would therefore appear to be the same. So it seems appropriate in the following analysis to pay particular attention to riboflavine and casein.

2. Increased Output of the Oxidized Decomposition Products Aminocresol and Aminobenzoic Acid, and Decreased Output of the Unchanged Product, Toluidine, on Supply of Protective Factors.

In these studies it was necessary to depart from the previous comparison between protective diet (HAMMARSTEN diet) and rice diet, as both contain uncontrolled amounts of vitamin B in the rice flour and, as far as the protective diet is concerned, in the casein. Instead of the above-mentioned protective diet, we have employed a basic diet, free from vitamin B, consisting of:

Vitamin-free wheat starch	636 g.
Peanut oil	57 "
Vitamin-free casein	220 "
Cod liver oil	3 "
Salt mixture	60 "
	<hr/>
	976 g.

The salt mixture consisted of:

Sodium chloride	52 g.
Magnesium sulphate	116 "
Sodium phosphate, monobasic	104 "
Potassium phosphate, dibasic	286 "
Calcium phosphate, monobasic	162 "
Calcium lactate	390 "
Ferricitrate	35 "
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	1,145 g.

To this diet were added aneurin (B_1), riboflavin (B_2) and nicotinamide (NSA) in such amounts that the animals received about 10 γ B_1 daily, about 40 γ B_2 , and about 600 γ NSA daily. As mentioned before, it was reasonable to assume that representatives of the B group might be expected to be present among the protective factors; and in order to avoid all too many possibilities of variations in the analysis, to begin with we included only B_1 , B_2 and NSA.

Insofar as an adequate diet — with regard to all the factors of the B group — was considered expedient, the animals were given an addition of yeast.

In the experiments the point was from the controlled protective diet to exclude some of the possibly active components and in this way to analyse their action. In order to have a uniform terminology for the text and figures in subsequent experiments, we have designated the combination of wheat starch, peanut oil, cod liver oil and salt mixture as *B-free diet*. If, in addition, the diet contains, for instance, vitamin-free casein, B_1 and NSA, it is designated as B-free diet + casein + B_1 + NSA.

A. Conditions on Supply of 2,3-Azotoluene.

First the analyses were aimed at the excretion of meta-aminobenzoic acid and aminocresol in rats which were given 30 mg. azotoluene daily (in oil solution) by mouth — by means of a finely graduated glass syringe with a blunt point. With the technique here employed, the same amounts of meta- and ortho-aminobenzoic acid give extinction values with a proportionality of 6 : 1. On administration of azotoluene, then, ortho-aminobenzoic acid may in a slight degree influence the values for meta-aminobenzoic acid (for particulars, see EKMAN, 1947 a). From *Figs. 2 and 3* it is evident that the excretion is considerably higher when the animals get B-free diet + B_1 + NSA + yeast + casein than when they only get B-free diet + B_1 + NSA + casein. The difference becomes even greater when one group of animals gets only B-free diet. Naturally, however, the difference here might have been the same also without this restriction of the deficient diet. The main difference in the beginning of the experiments may be taken to be the absence of B_2 in one group, and the findings in the latter part of the experiment may very well have resulted simply from the gradually increasing B_2 deficiency.

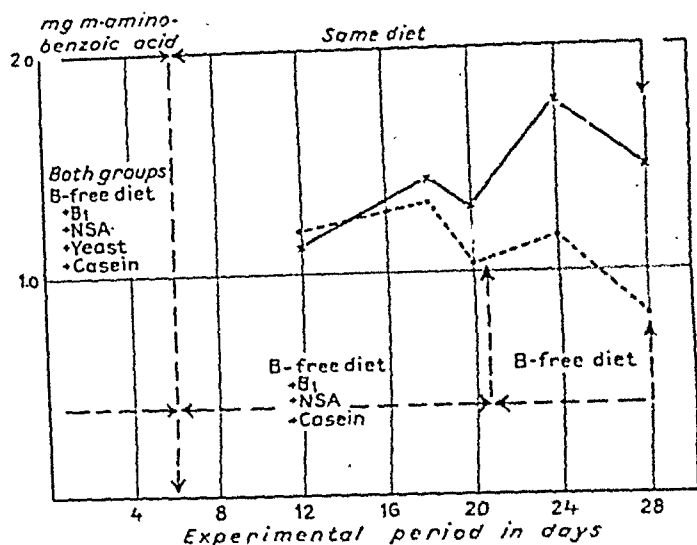


Fig. 2. Output of meta-aminobenzoic acid in the urine of rats which daily received 30 mg. 2,3-azotoluene by mouth. Each curve corresponds to the average values for 5 animals.

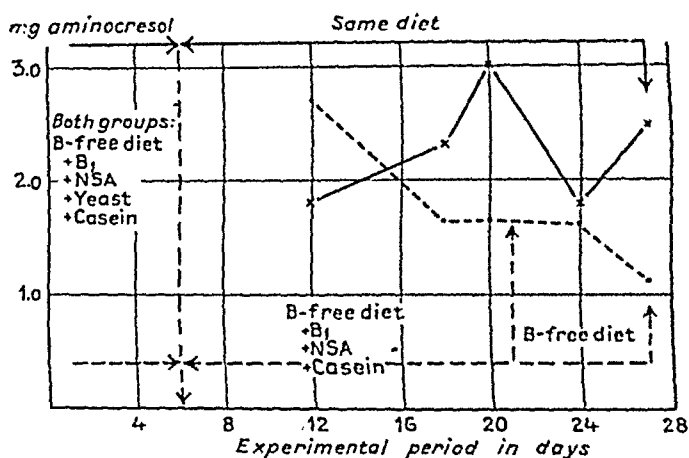


Fig. 3. Output of aminocresol. Same experiment as in Fig. 2.

B. Conditions on Supply of Meta-toluidine.

During a protracted experiment the meta-aminobenzoic acid output was determined throughout the experimental period, aminocresol and meta-toluidine through shorter periods. The animals were divided into two groups, each comprising 5 animals, all receiving a daily dose of 10 mg. meta-toluidine in oily solution

perorally. During a preparatory period both groups were put on B-free diet + B₁ + NSA + casein; and this diet gives B₂ deficiency. Then one group was given this diet with an addition of B₂, which according to *Fig. 4*, resulted in a quite considerable increase in the meta-aminobenzoic acid output, which subsequently fell

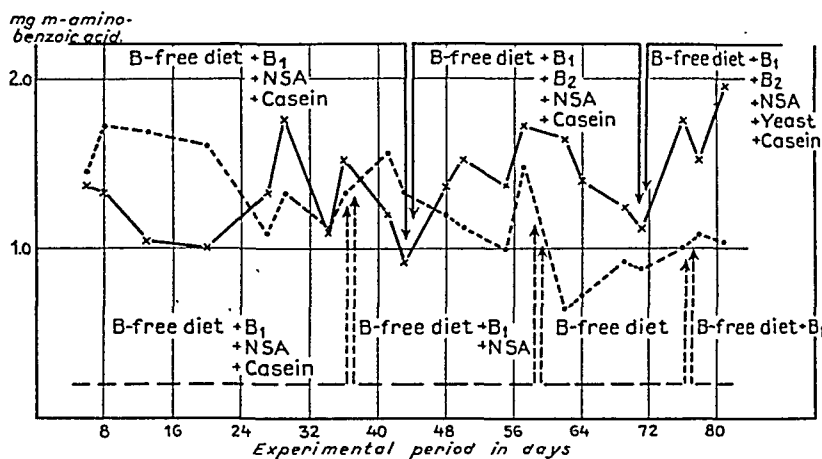


Fig. 4. Excretion of meta-aminobenzoic acid in rats which daily received 10 mg. meta-toluidine by mouth. Each curve corresponds to the average values for 5 animals.

off again. After this, an addition of yeast was given too, and this was followed by a very striking increase in the output. In the other group, casein was first excluded from the diet, later also B₁ and NSA. In the last week, when the animals showed symptoms of B₁ deficiency, this vitamin was again added to the diet. Largely, the meta-aminobenzoic acid output for this group showed a slowly progressing decrease.

Even though the variations in the output are somewhat irregular, it still seems justifiable to establish that very large differences in the output prevail between animals on the most adequate form of diet and animals on B-free as well as casein-free diets. It looks as if only the addition of B₂ would be able to increase the output. As in the azotoluene experiments, it cannot be decided conclusively whether the progressive decrease found for the deficiency group is due to the lack of B₂ alone or to some other factors too. The experiment is however continued.

During a certain part of the experimental period, determinations of aminocresol output were made too. As will be noticed from *Fig. 5*, there are considerable differences in the output

between animals receiving B-free diet and B₁, B₂, NSA and casein and animals receiving only B₁ NSA and B-free diet. Thus the difference is due to B₂ and casein; the rise in the curve for the group on the adequate diet, which previously had been wanting only B₂, may be due to the circumstance that this vitamin was added again.

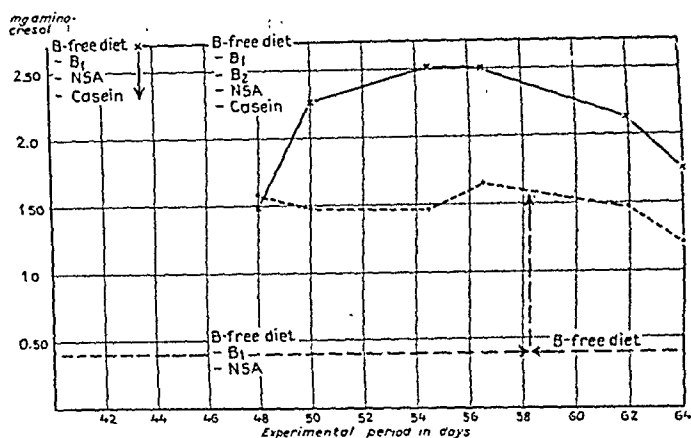


Fig. 5. Output of aminocresol. Same experiment as in Fig. 4.

As to meta-toluidine, the output of this could not be estimated throughout the experimental period on account of technical difficulties, but the results of the determinations performed are given in Table 1, where the number of experimental days refer to the

Table 1.

Excretion of Meta-Toluidine on Different Diets.
Same Experiments as in Figs. 4 and 5.

	Experimental period in days	55	69	71	76
Group I	Diet	B-free diet + B ₁ + B ₂ + NSA + casein			B-free diet + B ₁ + B ₂ + NSA + casein + yeast
	mg meta-toluidine	0.27	0.10	0.05	0.05
Group II	Diet	B-free diet + B ₁ + NSA			B-free diet
	mg meta-toluidine	0.56	0.43	1.15	1.10

values recorded in Figs. 4 and 5. It is plainly evident that on comparison between the adequate diet and the B-free the output on the adequate diet is practically nil, while on the B-free diet it is up to 1 mg. per day. From the description of the technique in a preceding paper (EKMAN 1947 a) the values cannot be looked upon as absolutely reliable. Each value recorded is the average for the 5 animals in the group, however, and as the found difference lies between 0 and 0.5—1 mg., the result may be considered established.

Thus, on certain experimental days, simultaneous determinations were made of meta-toluidine as well as aminocresol and meta-aminobenzoic acid, and therefore a balance can be set up with regard to the 10 mg. meta-toluidine received by each animal. We find then that in the presence of protective factors the estimated quantities amount to 1.5—2 mg. meta-aminobenzoic acid, 2—2.5 mg. aminocresol and 0 mg. meta-toluidine, whereas in the absence of protective factors the output is about 1 mg. meta-aminobenzoic acid, 1.5 mg. aminocresol and 1 mg. meta-toluidine. In both cases, the total output amounts to 35—40 % of the meta-toluidine supplies.

Isolation experiments now being carried out indicate that besides the excretory products of 2,3-azotoluene, which all contain an amino group, also products containing a carboxyl group but no amino group are excreted. Probably the latter products are formed on supply of meta-toluidine too, and they might explain in part the deficit in the balance. Furthermore, it is to be pointed out that the aminocresol value is calculated on the basis of a preparation which probably (EKMAN-STRÖMBECK 1947, and EKMAN 1947 a) contains ortho- and meta-aminocresol, while on administration of meta-toluidine, of course, only the latter form is excreted. So the absolute aminocresol value may possibly have to be corrected.

C. Conditions on Supply of Sulfanilamide.

During the preliminary experiments, before the work of the technique was concluded, tests were made also concerning another easily determinable — namely, sulfanilamide — on rice diet and rice diet with addition of riboflavine and casein. The animals were given sulfonamide mixed with the food. A certain amount of the diet was given daily, and the food not taken was collected every

day, dried and weighed. In this way it was possible to estimate the amount of sulfonamide received by the animals per day (on an average 40—50 mg.). Fig. 6 shows the daily output of sulfanilamide, expressed in percentage of the amount supplied (average of the values obtained from 8 animals). From Fig. 6 it is evident

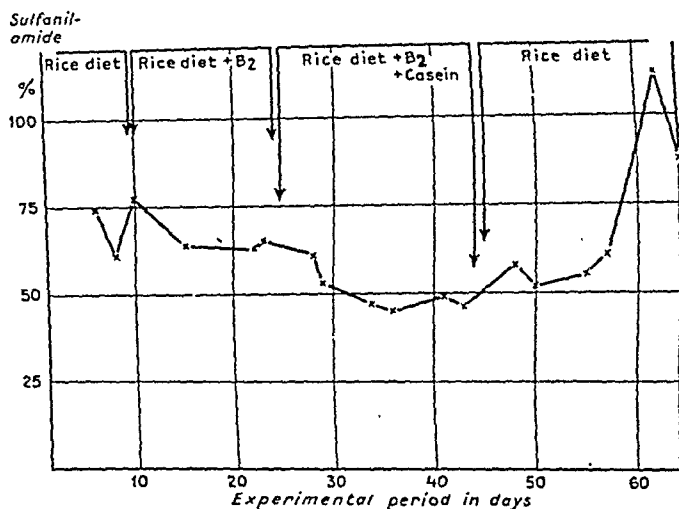


Fig. 6. Excretion of sulfanilamide in rats receiving on an average 40—50 mg. daily with the diet. The curve corresponds to the average values for 8 animals, giving the output in percentage of the ingested amount.

that while the animals on rice diet excreted about 70 %, the output decreases after addition of riboflavin and casein to the rice diet to 50 %, and on returning to the rice diet it increases to 100 %. The experiment, we think, is of interest because it could be demonstrated above that the protective diet brings about an *increased* output of the oxidized products of the compounds (azotoluene and meta-toluidine) supplied. In this experiment it now is found that — just as in the case of meta-toluidine — the excretion of the substance originally supplied *decreases* on addition of protective factors.

3. Mechanism of the Action of the Protective Factors.

A. Demonstration of Connection with the Ascorbic Acid Production in the treated Animals.

In the preceding section it was established that excretion of oxidized products of azotoluene and meta-toluidine increases,

above all, on supply of B₂, eventually also of casein, and at the same time the meta-toluidine output decreases. The same condition was found to apply to sulfanilamide. With our present know-

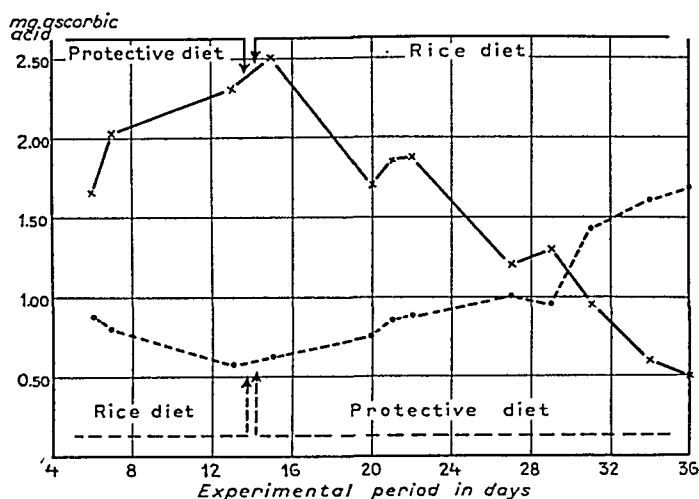


Fig. 7. Excretion of ascorbic acid in the same experiment as recorded in Fig. 1.

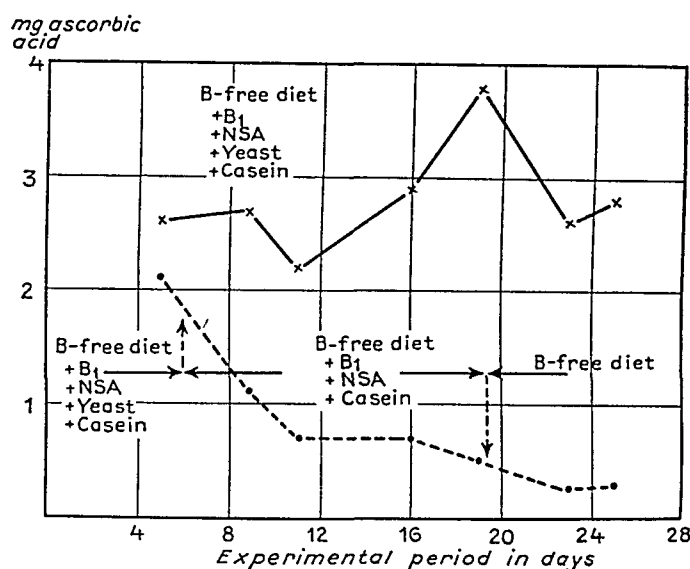


Fig. 8. Ascorbic acid output in the same experiment as recorded in Figs. 2 and 3.

ledge of the action of riboflavine and the effect of an adequate addition of protein it seems difficult to explain the effect here observed on the excretion of the aromatic compounds. In preceding works (EKMAN 1941, 1944), however, it has been shown

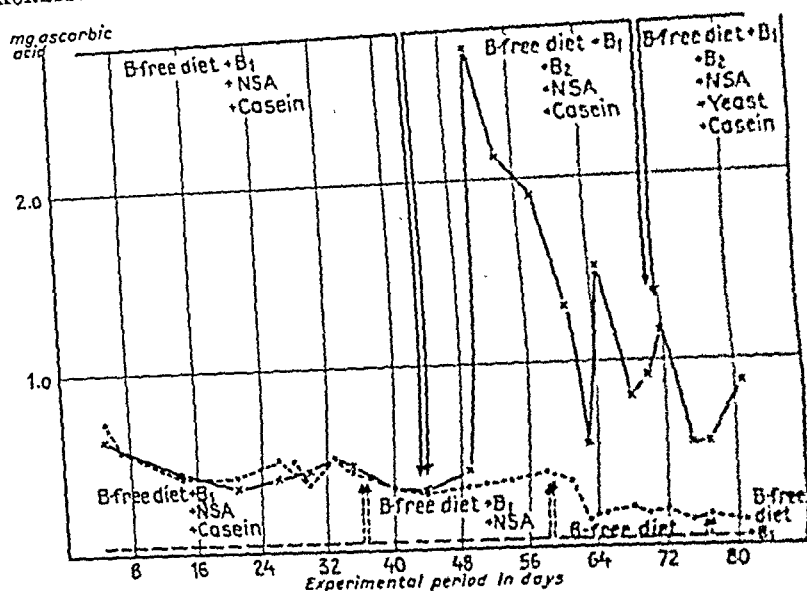


Fig. 9. Ascorbic acid output in the same experiment as recorded in Figs. 4 and 5.

that cyclic compounds are oxidized by ascorbic acid *in vivo* and *in vitro*. Certainly, the rats employed here for the experiments are able themselves to synthesize their vitamin C, and hence it does not look as if vitamin C would be able to play any rôle in the experiments here presented. Still, it may be mentioned that statements have been made in the literature about a connection between the synthesis of ascorbic acid in rats and simultaneous supply of vitamin B (*e. g.*, SVIRBELY 1936, SURE *et al.* 1939).

For this reason the ascorbic acid excretion was studied (as to the technique, see EKMÁN 1944) at the same time as the excretion of the aromatic products in the experiments reported above. From Figs. 7 and 8, which correspond to the excretion of the decomposition products of azotoluene in Figs. 1 and 2—3 respectively, and from Fig. 9, which corresponds to Figs. 4 and 5, in which the meta-toluidine experiments are recorded, it will be noticed that there is complete parallelism between the excretion of ascorbic acid and of the oxidation products: aminobenzoic acid and aminocresol. An increased excretion of these products is obtained only when the ascorbic acid output with the urine is increasing. Conversely, a decreasing ascorbic acid output is connected with an increase in the excretion of meta-toluene. As is evident from the preceding methodological studies (EKMÁN 1947 a), in all the cases the technique was elaborated so that the ascorbic acid in the urine

had no influence on the determination of the aromatic compounds. Furthermore, it can be emphasized that the not yet concluded experiments to ascertain which of the conceivable protective factors may be active here indicate that, above all, it is B_2 . *Fig. 10* shows plainly that an addition of casein to the diet has no effect

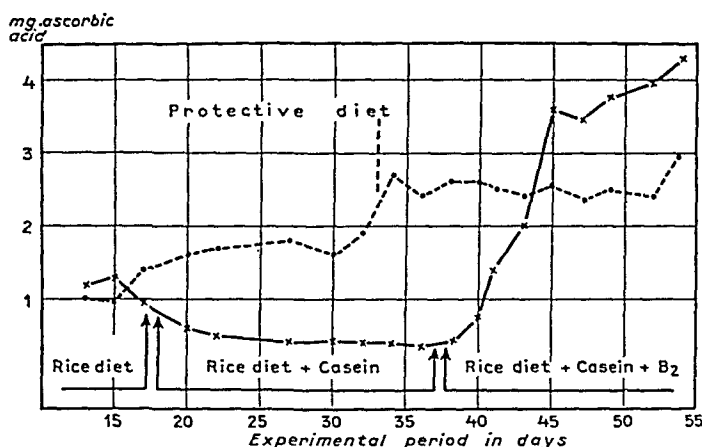


Fig. 10. Ascorbic acid output in rats on varying diets. Each curve corresponds to the average values for 5 animals. (Throughout the experimental period the animals received daily 30—45 mg. 2,3-azotoluene with their food.)

whatever on the ascorbic acid output even after three weeks, whereas an addition of riboflavine brought about a 6—10-fold ascorbic acid output already after one week. This indicates that the effect of the protective factors was attributable to B_2 , but the effect of B_2 might be indirect and ascribable to the influence which — according to these experiments — B_2 has proved to exert on the ascorbic acid production in rats. In turn, ascorbic acid would then — through its oxidizing cleavage of the aromatic compounds — afford the explanation of the results obtained.

In order to prove this assumption, however, it should be possible, of course, to replace the B_2 effect by supply of ascorbic acid in B_2 deficiency. As will be noticed from *Fig. 11*, indeed, this is also possible. *Fig. 11* shows that in a group of 8 rats, kept on the rice diet, the excretion of sulfanilamide lies at a high level. On administration of 20 mg. ascorbic acid by injection (which gives excretional values for ascorbic acid that are comparable to the highest values obtained in the previous experiment), the excretion falls to about one-half, and it rises again on discontinuance of the ascorbic acid supply. Naturally this result has to be established also for azotoluene and meta-toluidine, and experiments are

now going on in order to demonstrate a tumour-preventive effect of ascorbic acid against azotoluene in animals placed on a deficient diet, as well as to reproduce the effect obtained on the excretion of sulfanilamide.

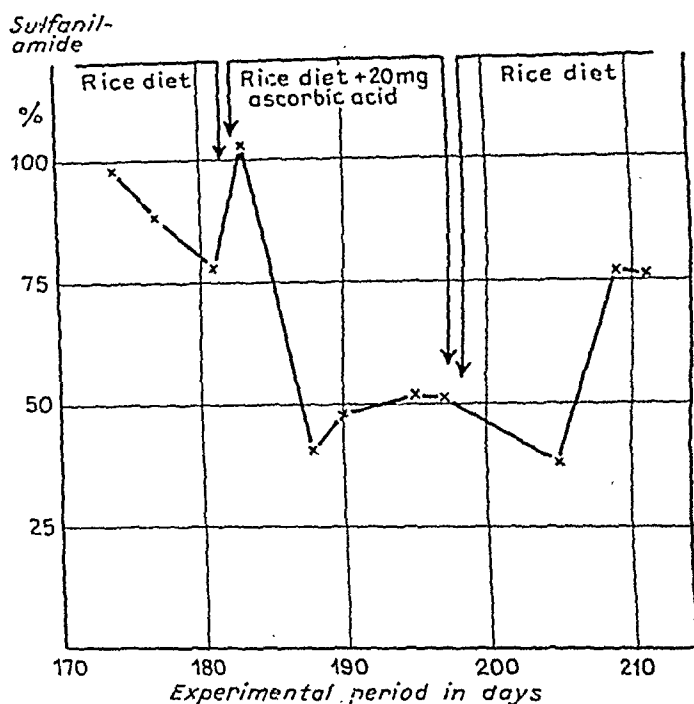


Fig. 11. Sulfanilamide output in rats on daily ingestion, on an average, of 40—50 mg. with the food. The curve corresponds to the average values for 8 animals, giving the excretion in percentage of the amount ingested.

The variations observed in the ascorbic acid output may be due either to a change in the conversion of a continually constant amount of synthesized ascorbic acid or to a change in the capacity for the synthesis of this substance. That the latter probably is the case will be shown in a subsequent paper (EKMAN 1947 b).

B. The Protective Factors examined have no Influence on the Course of the Acetylation in the Organism.

The effect of the protective factors might be due partly to a cleavage of the tumor-producing compound, partly to its influence on the excretion of this substance in combined form. As to the acetylation, it seems reasonable to reckon that the same conditions

that influence the acetylation of an aromatic amine exert their influence on another amine too. In the experiments here presented concerning sulfanilamide, the ratio between free and acetylated sulfanilamide was determined at the same time. It was then found that neither B₂ nor ascorbic acid, nor the change from the protective diet to the rice diet, had any influence whatever on the ratio between free and acetylated sulfanilamide. In all the experiments this ratio has been particularly constant, about 1 : 1.

4. Significance of the Results presented to the Explanation of the Latent Period preceding Demonstrable Changes in the Mucous Membrane in the Animals given Azotoluene.

OTSUKA & NAGAO (1936) obtained tumours only in the animals that survived the treatment for 122 days or longer, and STRÖM-BECK obtained papillomatous proliferation only on treatment of the animals for 100 days or more. Animals on which autopsy was performed earlier showed no changes in the bladder. So it requires a certain, not inconsiderable, latent period before any changes may be observed.

Of course, this has its natural explanation in part in the fact that the very early changes are not noticeable. But it also seems conceivable that, under the given experimental conditions, a part of the latent period is necessary for the animals to reach the state of deficiency, which obviously is essential to the development of pathological changes.

This assumption appears to be in keeping with the observation reported by MAISIN et al. (1941), that in rats given butter yellow as addition to a diet consisting chiefly of polished rice, hepatoma appeared in some cases as early as after 90 days, and at a relatively high frequency after 180 days, whereas in rats on a diet consisting chiefly of rye flour the first hepatomas did not appear till after 240—300 days.

It may be mentioned that 9,11-dimethyl-1,2-benzanthrene painted on the skin produces tumors already after 43 days.

The observation reported in Section 2, that the meta-toluidine output in the presence of protective factors practically is nil, whereas in the absence of such factors a distinctly demonstrable excretion takes place, is of considerable interest. Apparently it takes a certain length of time before rats on the rice diet excrete

any meta-toluidine with the urine at all, even when the animals are given this substance by mouth, so that only after this period can the required contact take place between the tumorigenic element in the urine and the mucous membrane of the bladder.

Experiments with meta-toluidine aimed at this point are not yet carried out. But *Fig. 12* shows that only after 60 days, did the rats (10 animals) which received sulfanilamide (40—50 mg. per day) show an excretion of sulfanilamide rising to about 100 %

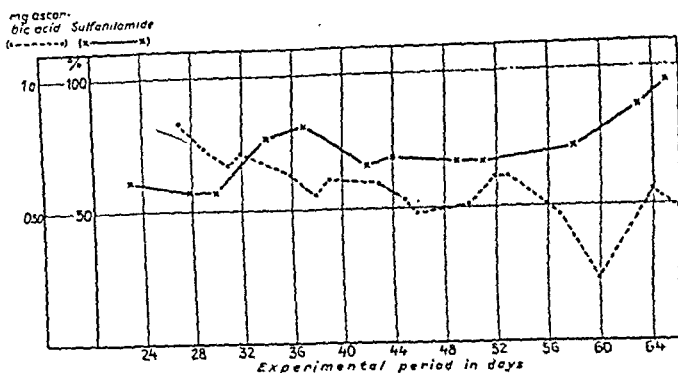


Fig. 12. Excretion of ascorbic acid and sulfanilamide in rats receiving daily 40—50 mg. sulfanilamide with their food (rice diet). Both curves correspond to the average values for 10 animals. The ascorbic acid output is recorded in mg. per day, the sulfanilamide excretion in percentage of the amount ingested per day.

of the amount supplied, suggesting that the protective factors available had been used up. *Fig. 12* further shows that during this period the output of ascorbic acid decreases slowly. This experiment may possibly illustrate the idea here presented, but it can be proved, of course, only if an excretion of meta-toluidine on supply of this substance to animals on the rice diet is found to appear only after a certain length of time. If so, it also ought to be practicable to promote this phenomenon by employment of an entirely B_2 -free diet instead of the rice diet — and thus obtain the mucosal changes in the bladder within a period considerably shorter than 100 days.

This train of reasoning might be of interest also in another connection. It is well known that workmen in the dye industry who are occupied with the production of aniline to some extent are liable to tumours of the bladder. So far, however, it has not been possible experimentally to obtain aniline tumours by giving animals aniline by mouth. On the other hand, it is to be pointed out

that YAMASAKI and SCHOICHI (1937), who introduced an aqueous solution of aniline directly into the bladder on animals, obtained the development of epithelioma. In view of our meta-toluidine experiments it seems conceivable that in the attempts through oral administration of aniline to produce tumours the animals were on such a diet that their vitamin requirement was covered, and no aniline output with the urine ever took place.

Summary.

The experimental results obtained in this work and in preceding studies (EKMAN and STRÖMBECK 1947) may be summarized most simply as done in the following tabulation.

Tumorigenic compound	Excretory products	Output	
		Presence of protective factors in the diet	Absence of protective factors in the diet
2,3-azotoluene	aminobenzoic acid, aminocresol, toluidine	high high —	low low —
meta-toluidine	aminobenzoic acid, aminocresol, toluidine	high high very low or nil	low low high
Ascorbic acid		high	low
Sulfanilamide		low, same effect from ascorbic acid	high
Sulfanilamide		50 %	50 %

The protective factors are assumed, above all, to correspond to B₂. Still, some effect from other vitamins belonging to the B group cannot yet be excluded.

The influence of the protective factors on the excretion of the tumorigenic compounds is interpreted as a secondary result of their influence on the synthesis of ascorbic acid.

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A Cutaneo-Muscular Reflex in Man.

By

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In a previous investigation BING, CARLSTEN and CHRISTIANSEN (1945) measured muscular temperatures during cooling of a limited area of the skin with ice. After approximately 7 minutes of cooling a decrease in temperature was observed, which varied considerably in different individuals. It could be demonstrated that this decrease is caused by an increased loss of heat from the muscle and not by a decrease in blood supply due to a vasoconstriction. In some cases the fall in temperature was preceded by a transitory rise. As the rise was depressed, when the blood supply to the muscle was blocked, it was assumed that it is due to an increase in intramuscular blood flow. The present paper deals with a further investigation of the mechanism of the transitory rise.

Technique.

The intramuscular temperature is recorded by means of a thermocouple of the type used by BUCHTHAL et al. (1944). The temperature is measured in the m. biceps brachii and m. rectus femoris during and after exposure of the skin to different temperatures (0—60° C.). During the experiment the temperature of one junction is kept constant at a value between 28 and 35° C. The other junction is placed in the muscle 30 mm below the skin. Special care is taken to use only thin thermoneedles, which have a low heat capacity and cause the least damage to the tissue. The inconstant occurrence of the transitory rise in the former experiments (BING et al. 1945) was probably due to the use of needles with relatively high heat capacity. The needles applied are 0.5 mm thick and 30 mm long.

The thermocurrent is recorded by a light-spot galvanometer (multiflex) with a sensitivity of 1° scaledivision $= 3.5 \times 10^{-9}$ Amp. and an internal resistance of 1196Ω . Temperatures are registered with an accuracy of 0.05°C . The local variation of the skin temperature is obtained by applying 2 blocks of ice with a cooling area of 16 cm^2 each or 2 copper containers of the same size filled with water of different temperatures. They were placed on the skin directly or wrapped in thin rubber bags, and the thermoneedle was inserted between the 2 ice blocks or containers.

In control experiments it was ascertained, that only the temperature of the junction at the tip of the needle influences the thermocurrent. Temperature changes on other parts of the needle proved to be without effect. It could further be shown that the effect is not due to minute displacements of the needle caused by the local application of the ice, as a corresponding pressure exerted by containers of skin temperature does not produce any rise. In model experiments on meat, which was kept in a water bath at 34°C . and covered with fresh human skin the transitory rise never occurred and only a continuous fall in temperature was observed.

Results.

The rise in temperature which precedes the decrease amounts to $0.7\text{--}1.6^\circ \text{C}$ and occurs after $15\text{--}30$ seconds of cooling. Its size depends on the distance between the area which is cooled and the position of the thermojunction. From Fig. 1 it can be seen that the smaller the distance the higher the rise in temperature. With a distance of more than $4.6\text{--}5 \text{ cm}$ between the centre of the cooled area and the thermojunction no increase in temperature occurs. When the ice block is removed after the transitory increase in temperature, the temperature decreases with a steep gradient, while it shows a continuous fall when there has been no previous rise in temperature. The steep fall may happen at any part of the curve when the cooling is removed after the rise has been passed. Experiments with cooling lasting 1, 2, 3, 4, 5 and 12 minutes showed that a maximum increase in temperature was reached after $3\text{--}4$ minutes. If the cooling lasted less than $3\text{--}4$ minutes the increase was smaller, depending on the duration of the cooling period.

It could be demonstrated that the rise in temperature is not caused by a local activity of the muscle. When concentric needle electrodes are placed close to the thermoneedle no action potentials can be detected even when maximum amplification is applied. The rise in muscle temperature which follows voluntary contrac-

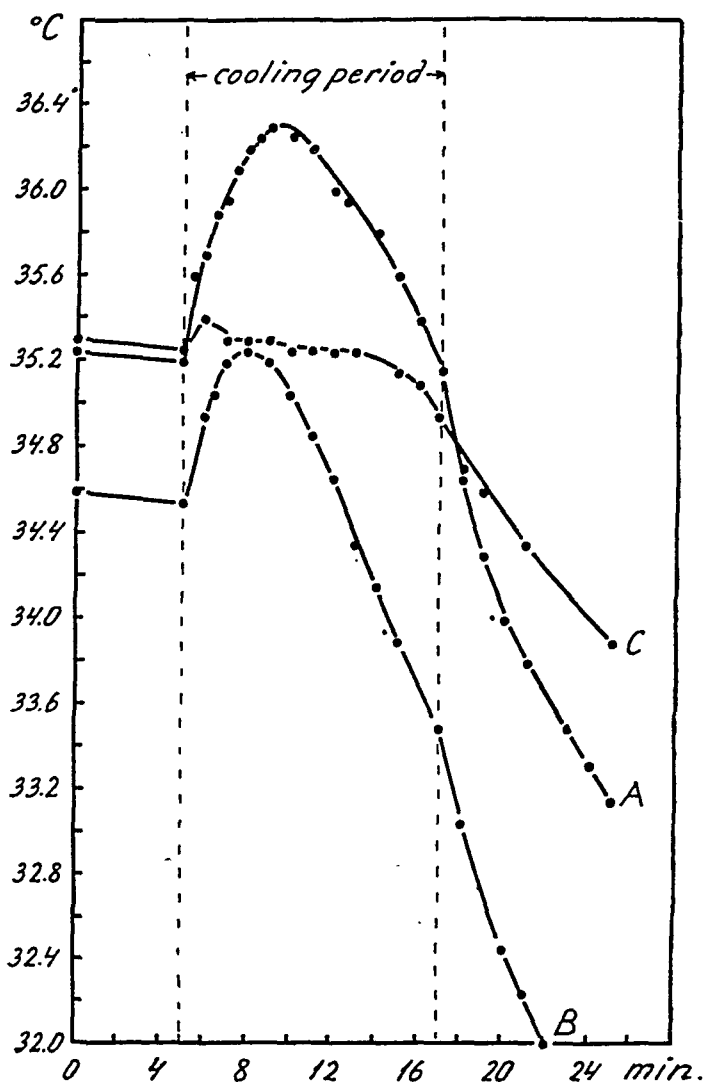


Fig. 1.

The influence of the distance between the middle of the cooled area and the thermojunction on intramuscular temperature.

Curve A: distance 37 mm

» B: » 40 mm

» C: » 47 mm

The rise in temperature increases with decreasing distance. The decrease after the initial rise becomes steeper, when the cooling stops.

Abscissa: time in minutes.

Ordinate: muscle temperature in °C.

tions (BUCHTHAL et al. 1944) takes double the time as that accompanying cooling.

The temperature course after local arresting of the circulation

was examined in 2 persons. The brachial artery was blocked by means of an elastic rubber band around the arm for a period varying between 5 and 28 minutes. In contradiction to the observations described by BING et al. (1945) the temperature also increased during cooling with arrested circulation, but the magnitude of the rise decreased with the duration of the preceding time

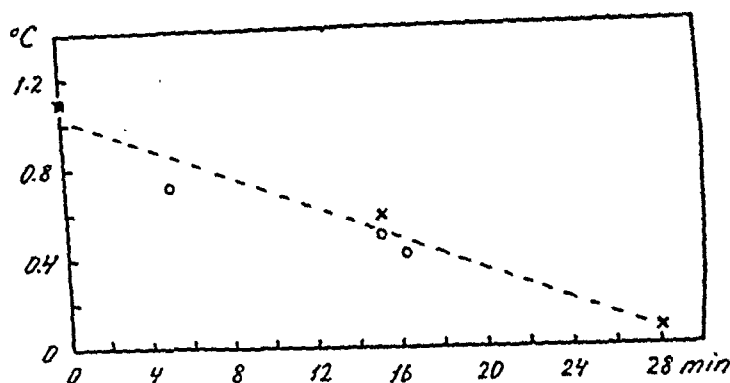


Fig. 2.

The rise in temperature during cooling after arresting of the circulation.
 Abscissa: Duration of the period with arrested circulation in minutes.
 Ordinate: Rise in °C.

Person A. A. ○

» G. S. +

of blockade (Fig. 2). This finding is rather unexpected and may either be due to ineffective arresting of the circulation (perhaps due to anastomotic flow from intraosseal blood vessels) or to local displacements of the blood in the extremity due to localised vasodilatation.

In order to investigate, whether the increase in temperature is due to a spinal reflex mechanism, experiments were performed on 3 persons under spinal anesthesia. Even during complete motor and sensitive paralysis a perceptible rise of the muscle temperature with a subsequent fall was observed as a result of local cooling.

In 3 experiments performed on 2 persons 5 ml 0.5 per cent novocaine (without adrenalin) were injected intramuscularly 8, 12 and 13 minutes before the cooling with ice. In one case there was no rise at all and in the others it was reduced to 0.2° C. Injection of a corresponding amount of Tyrode solution had no effect.

Injection of 0.5 ml histamine subcutaneously and close to the needle causing a strong flush of the skin, is not accompanied by a change in muscle temperature. Local cooling of the skin 10 minutes later produces the usual rise.

The rise which is obtained when copper containers with water at 15—18° C. are used for cooling is smaller than that obtained with ice. It amounts to 0.3° C. When high temperatures up to 60° C. are applied no initial rise in intramuscular temperature is observed, even if pain reactions are produced.

The initial muscle temperature is without influence on the course of the cooling reaction in normal individuals. In experiments on 2 patients suffering from paresis after polyomyelitis with a low temperature in the paretic muscles (31 and 33° C. respectively) the initial rise in temperature which accompanies cooling was found to be the same as that in normal persons. It is also present in cases of Buerger's disease (2 patients), claudicatio intermittens caused by arteriosclerosis (2 patients) and in one case of disturbances after embolism of the popliteal artery. The temperature of the muscles in these cases was between 30 and 32° C.

Discussion.

The present observations indicate that the primary rise in temperature which accompanies local cooling occurs rather constantly and is independent of the muscle temperature at the beginning of the experiment. It is not due to muscle activity produced by the cooling, and since a decreased heat dissipation through convection or radiation is excluded, it can only be explained by assuming an increased heat supply through the blood flow. It is in accordance herewith that the rise is considerably smaller, when the circulation to the extremity under examination is interrupted. The fact that the reaction occurs very early in the cooling period and is reduced or abolished by local intramuscular application of novocaine indicates a neurogenic release of the phenomenon. This assumption is supported by the histamine experiments. When histamine is injected subcutaneously in relatively large quantities, the muscle temperature remains unaltered.

It was rather surprising that spinal anesthesia did not affect this temperature reaction. It indicates that the rise may probably be due to changes in the blood supply which are released by an axon reflex initiated by the stimulation of sensory receptors in the skin or the underlying tissue. Axon reflexes in the skin have been described in man by LEWIS (1927), but as far as we know, such reflexes from skin to muscle have not been demonstrated

hitherto. The fact that the reaction is not produced by local treatment of the skin with heat indicates, that the receptors are cold receptors. The increased rapidity of the fall of temperature which was observed when the cooling was removed makes it reasonable to assume that the rise is caused by an active dilatation of the muscular vessels.

Summary.

The transitory rise in the temperature of the underlying muscles, which follows local cooling of the skin is due to vasodilatation, probably released through an axon reflex.

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Regulation of Respiration during Muscular Work, as studied on the Perfused Isolated Head.

By

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Received 8 May 1947.

In the discussion about the regulation of respiration during muscular work two main theories have been put forward. According to the classical view the determining factor is to be sought in the blood itself, attention being focussed on its concentration of carbon dioxide and hydrogen ions as well as on the degree of desaturation of the oxyhæmoglobin. A number of investigators have seen as the dominating influence on respiration during muscular work, nervous impulses irradiating from the motor cortex or from the muscles themselves. It is not necessary in this connection to enter into details, some recent work having been reviewed earlier (EULER and LILJESTRAND 1946). It is obvious, however, that the two different theories are not necessarily irreconcilable. It is well known that sensory stimulations may profoundly influence respiration, and there is no reason why this should not be possible also during muscular work. But it seems difficult to understand that such a mechanism could be the determining factor and be responsible for the usually rather close correlation between oxygen consumption and ventilation. It would seem simpler to assume that the extent of the metabolism itself by intermediation of the blood should constitute the fundamental regulation, and that other factors would add their influence to this basic regulation and thereby modify the result more or less.

The situation is complicated by the fact that oxygen want and carbon dioxide accumulation in part have different points of attack, oxygen want stimulating respiration reflexly from the carotid and aortic bodies but usually having a depressing action directly on the centre, whereas increase of the carbon dioxide tension (and of the hydrogen ion concentration) will stimulate respiration directly as well as reflexly. Their relative influence on the final result may also vary considerably. The results obtained in different cases will therefore often be difficult to interpret. Thus, *e. g.* oxygen want sometimes leads to an overventilation with washing out of carbon dioxide so that the carbon dioxide tension and the hydrogen ion concentration are lowered, as found especially when the oxygen percentage is reduced in inspired air (cp. BJURSTEDT 1946). Stimulation of sensory nerves may act in the same direction. Especially when small or moderate amounts of muscular work have been performed, these different factors may become of great importance and even dominate the picture more or less. It is noteworthy that in experiments on man recent investigators have come to the conclusion that during light and moderate work the regulation of respiration is achieved reflexly from the working muscles (ASMUSSEN, CHRISTENSEN and NIELSEN 1943, ASMUSSEN, NIELSEN and WIETH-PEDERSEN 1943) whereas during heavy work the composition of the arterial blood is supposed to be of primary importance (ASMUSSEN and NIELSEN 1946). Experiments on cats and dogs performed by EULER and LILJESTRAND (1946) led to the conception, however, that also during moderate work the main effect on ventilation is due to influences acting through the blood. After elimination of the carotid and aortic bodies the increase in ventilation during work was smaller than before, but there was still a considerable effect so that the blood was supposed to act both on the centre and the chemoreceptors.

The difficulty of separating the different factors through the cooperation of which respiration is regulated during muscular work made it desirable to study the problem with the aid of the isolated perfused head. In this way it would be possible to obtain definite information whether the arterial blood during moderate work is altered in such a way as to stimulate directly or indirectly the respiratory centre to increased activity, and it might also to some extent be possible to differentiate between the chemical factors concerned.

Methods.

The technique for the perfusion of the head of one dog from another dog has been described in detail elsewhere (HEYMANS and HEYMANS 1926), so that it will be sufficient to give here only an outline of the

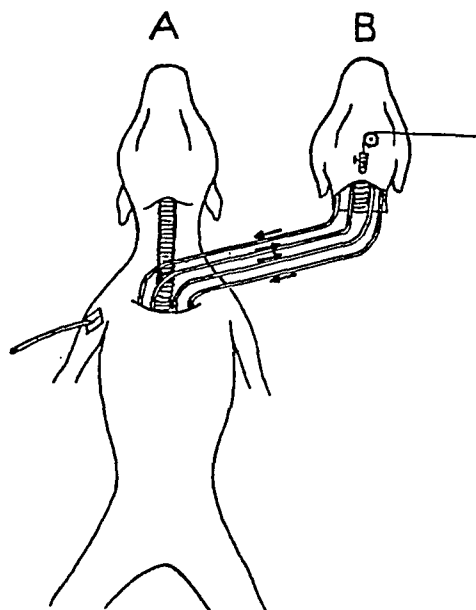


Fig. 1. Scheme of perfusion of the isolated head of dog B from dog A.

principles of the procedure. Two dogs (fig. 1), A and B, are anesthetized. Dog A serves as the donor and ought to be greater than dog B. The cephalic ends of the two carotids and of the two external jugular veins of B are united with the cardiac ends of the two carotids and the two external jugular veins respectively of dog A. With a special instrument the head of B is then completely separated from the rest of the body at the level of the 4th or 5th cervical vertebra. The circulation between A and B is now established, and respiration of the perfused head soon starts. We recorded the respiratory movements of the perfused head from the larynx in the way described by HEY-

MANS and HEYMANS simply by a thread that was led over wheels to a lever (preparations I, II and IV) or in a corresponding way from the nostril (preparation III). In the donor dog the ventilation was measured at intervals, the tracheal cannula being connected with LOVÉN valves and a gas meter; in one preparation the respiratory movements of the donor dog were recorded by a Marey pneumograph (preparation IV). Blood pressure was recorded from the brachial artery of the donor dog. Since the blood pressure regulation in the donor after joining both carotids with the corresponding vessels of dog B became greatly impaired, in the following preparations only one carotid artery of the donor dog was united with the head of dog B, both external jugular veins still being handled as mentioned above. In this way a sufficient perfusion was obtained, and the control of the blood pressure in the donor remained in a good condition. Anesthesia was established on the donor dog by chloralose (0.08—0.1 g per kg) in preparations I, II and IV or by urethane (6.6 ml of a 25 per cent solution) in preparation III and by ether in dog B.

Electrical stimulation of the donor dog was obtained by an apparatus ("Innervator") which could give tetanizing or "modulated" currents of varying strength and duration. The different electrode was placed under the skin of the back over the sacrum and the indifferent electrode in preparation I under the skin of the lower part of the abdomen and in the preparations III and IV on one of the legs. At the closing of the current both legs were then stimulated.

In all, perfusion of the isolated head was arranged in four preparations. In one of these (II) the donor dog was breathing badly, so that artificial respiration had to be given at intervals. This preparation therefore could not be used for experiments with muscular work but only to try the effects of different ventilations of the donor and the accompanying reactions of the perfused head. In the other three cases the donor dog as well as the perfused head were in good condition, and a number of determinations during work could be performed. Since the general agreement was excellent we have considered it unnecessary to extend the material further.

Results.

1. Overventilation and Muscular Work.

If artificial respiration was applied to the donor dog, it could easily be demonstrated that overventilation rapidly led to a decrease of the respiratory rate as well as the amplitude, in the perfused head. This is of course to be expected, since the perfusing blood became more alkaline and the oxygen saturation of its hæmoglobin probably increased somewhat. Thus a reduction had taken place with regard to the two factors which normally stimulate respiration during rest, the carbon dioxide tension and the desaturation of the oxyhæmoglobin of the arterial blood.

In order to obtain a fairly large increase in oxygen consumption and ventilation in the donor dog, our first experiments with muscular work in preparation I started with a very strong tetanizing stimulation, repeated at a rate of about 27 per minute. The consequence was that ventilation rose from a resting value of 2.4 to 8.5 liters per minute and probably even more. The respiratory movements of the perfused head, however, showed a decreased amplitude during the working period and to some extent also after it. Fig. 2 illustrates these results. The experiment was performed five times with the same kind of stimulation but of varying duration. The following table summarizes the results obtained.

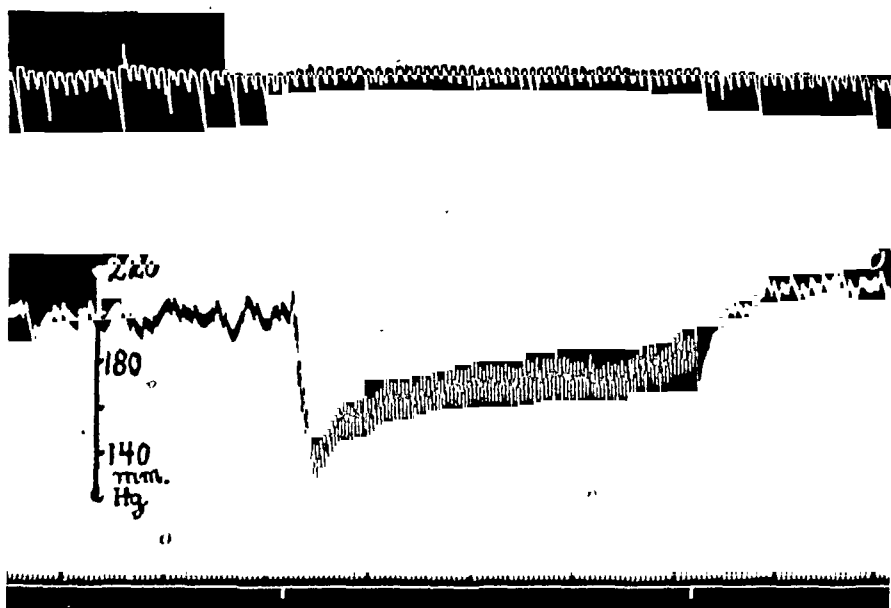


Fig. 2. Determination 4 (Preparation I). Donor dog (A) 14 kg., dog B 9 kg. Head of B perfused from the two carotids and the two external jugular veins of A. Curves represent from above respiratory movements of perfused head, arterial blood pressure of A, time in minutes and periods of 3 seconds, signal marking. Between marks stimulation of A with tetanizing currents about 27 times a minute. Cp. table 1.

In this and the following tables +, — and 0 signify increase, decrease or no alteration respectively of amplitude in relation to resting value before work.

It turned out that each stimulation was followed by a deep respiration of the donor dog, probably owing to a direct action on the diaphragm and that therefore a considerable overventilation had occurred. The decreased amplitude of respiration of the perfused head will therefore easily be explained as the result of washing out of carbon dioxide. As seen from the table there is, however, a certain tendency to increased respiratory rate in the perfused head during and after work in the donor dog, as the duration of the period of stimulation becomes longer. This is clearly demonstrated by a comparison between determinations 2, 3 and 4. The reason for this increase which was not observed during simple overventilation is probably a certain amount of oxygen want — which is known usually to increase the rate of respiration. During the contractions the blood flow to the working muscles was probably more or less restricted and during the phase of relaxation the

Table I.

Respiratory movements of perfused head during tetanizing stimulation about 27 times per minute of donor dog.

Number of determination	Duration of stimulation min.	Rate per min. before work	Rate per min. during work	Amplitude during work	Rate per min. after work	Amplitude after work
1	1	16.0	16.0	—	16.0	—
2	2	8.0	6.1	—	7.3	—
3	3	8.1	9.1	—	10.8	—
4	4	9.5	11.5	—	10.2	—
5	3	11.0	12.0	+	11.1	+ Resistance increased in donor dog by valves and gas meter

complete restoration may have been difficult. A certain indication of oxygen want could be found in the behaviour of the blood pressure of the donor dog: during the working period it rose slowly and after the end of the work it attained a level that rose with the duration of the working period to respectively 182, 197 and 213 mm. in determinations 2, 3 and 4. In determination 5 the donor dog had been connected to respiration valves and a gas meter with a considerable resistance. We are inclined to believe — and this view is strengthened by direct observations on our other preparations — that this has somewhat reduced the overventilation with the result that now not only a quicker rate but also a slight increase in the amplitude of the respiratory movements of the perfused head was observed.

The results seem to show that even during overventilation a certain stimulation of the respiration of the perfused head was transmitted by the blood from the working animal. This stimulation seems to be connected with oxygen want.

2. Different Kinds of Muscular Work during Air Breathing.

A. Tetanizing Currents.

Since the results mentioned above were interpreted as due to a considerable overventilation during the working period, the stimulation was modified in different ways. In preparation I the same

strong tetanizing currents as mentioned above were used but stimulations were made less frequent and at the same time of longer duration. Thus in one experiment (6) contractions were induced for periods of 6—7 seconds and followed by resting periods of 3—4 seconds. In four further determinations on this preparation each stimulation lasted 10 seconds and the resting period 5 seconds. In this way overventilation, if it occurred at all, was at least greatly reduced, since electrically induced contractions of the diaphragm in dog A of this preparation were restricted to 10 and 4 respectively per minute. Ventilation rose from the resting value of 3 to about 6 liters per minute, thus considerably less than in the experiments quoted under 1. In the experiments on preparations III and IV the indifferent electrode, as mentioned above, was placed on one leg, thus eliminating the risk of simultaneous contractions of the diaphragm. The duration of each stimulation and resting period varied somewhat in the different determinations, the details being given in table 2, which comprises all our determinations with tetanizing currents with the exception of those given in table 1. The amount of work performed was very moderate in all cases, the ventilation of the donor dog usually rising at most to about twice the resting values.

As seen from table 2 there was in every experiment with one exception (8) a marked increase of the rate and the amplitude of respiration of the perfused head not only during work but also after it. Fig. 3 and 5 afford typical illustrations and demonstrate an intimate correlation between the ventilation of the donor and the respiratory movements of the perfused head. A closer scrutiny reveals some interesting features. The longer the periods of contraction in comparison with the rest between the stimuli the more pronounced was the increase after work. This is clearly seen *e. g.* in preparation III, where stimulation for 5 and rest for 5 seconds each produced a definite rise of the rate and deeper breathing during work but only an effect on the rate, with hardly an increase of the amplitude, after it. If each stimulus was made to last 10 seconds followed by a period of 5 seconds rest, a similar result was obtained during work, but the great effect was found after it. In a corresponding way in preparation IV the strongest after-effects were observed with stimulations of 15 seconds duration, followed by 5 seconds rest, then came stimulation 10 seconds with 5 seconds rest and a further reduction in the after-effect took place when both periods had a duration of 10 seconds. The

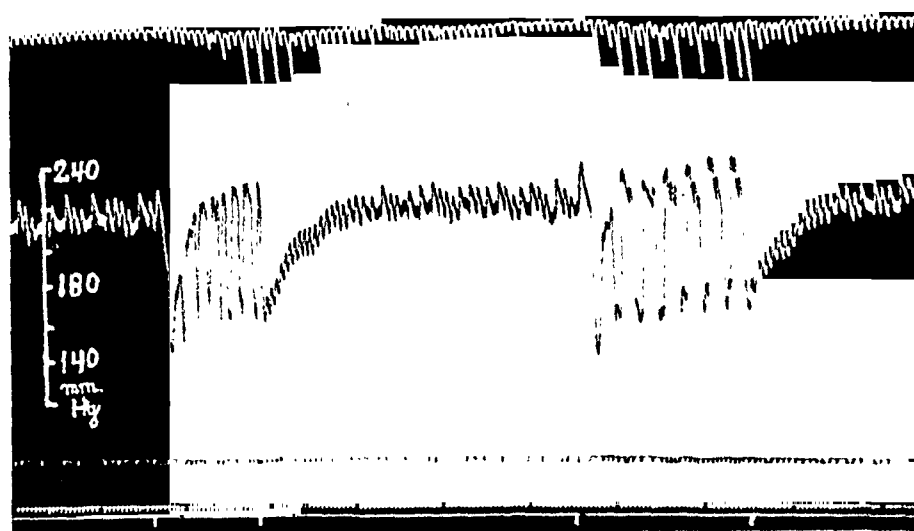


Fig. 3. Determinations 6 and 7 (Preparation I). Designations as in fig. 2. The curve above the time-marking gives the ventilation of dog. A. (Between the marks 0.1 liter.) Between marks electrical stimulation of A with tetanizing currents (about $7'' + 3''$ and $10'' + 5''$ respectively). Cp. table 2.

explanation seems to be the same as mentioned above, the restitution being less complete, as the period of relaxation was shortened in comparison with the duration of the contraction. Thus the greater the static component of the work, the more pronounced was the increase in respiration after the cessation of work, whereas the effect during work had a certain tendency to become less evident. LINDHARD (1920) has shown in man, that prolonged static work may be accompanied only by a moderate increase in ventilation with a great effect after the work has finished. In his cases the work (hanging in the bent arms) was prolonged as much as possible (up to 0.9 minutes), but it seems evident that there is a varying amount of fairly long-lasting contractions in many of our usual movements. Our results are in good agreement with his and show that this after-effect as well as the effect during work must, to a great extent, be ascribed to alterations in the chemical composition of the arterial blood.

There are also individual differences to be seen from table 2. Preparation III exhibits a greater tendency to after-effects than does preparation IV, though the arterial blood pressure in both cases remained at about the same level throughout the determina-

tions (120—150 for preparation III and 130—150 for IV). It may be mentioned, however, that in III the donor dog had been anesthetized with urethane and in IV with chloralose.

The experiments referred to in this section leave no doubt that during and after moderate muscular work of different kinds, respiration is stimulated to a great extent through the blood.

B. Modulating Currents.

In order to decrease the static factor during the working periods it was considered desirable to make some experiments with so-called modulating currents, which seem to be more similar to the usual nerve impulses than the tetanizing currents. The modulating currents increase relatively slowly and decrease in a corresponding way. Table 3 shows the results obtained in this way (all from preparation IV), and fig. 4 gives an illustration. The parallelism between the respiratory activities of dog A and the perfused head is striking. A comparison with the determinations 19, 23 and 25 reveals that the effects during work are relatively greater in comparison to the after-effects when modulating currents were used. The results seem to be in good agreement with the interpretation given above.

Table 3.

Number of determination	Duration of stimulation min.	Stimulation periods per min.	Respiratory movements of the perfused head				
			Rate per minute before work	Rate per minute during work	Amplitude during work	Rate per minute after work	Amplitude after work
20	3	8	12.1	11.1	++	11.1	+
21	3	20	11.6	12.2	++	12.6	0
22	3	20	15.9	16.9	++	16.9	0
24	3	8	16.9	19.0	++	17.6	+

3. Influence of Oxygen Inhalation.

The assumption that oxygen want plays a rôle as one of the factors concerned with the stimulation of respiration in our work experiments led to similar determinations when the donor dog

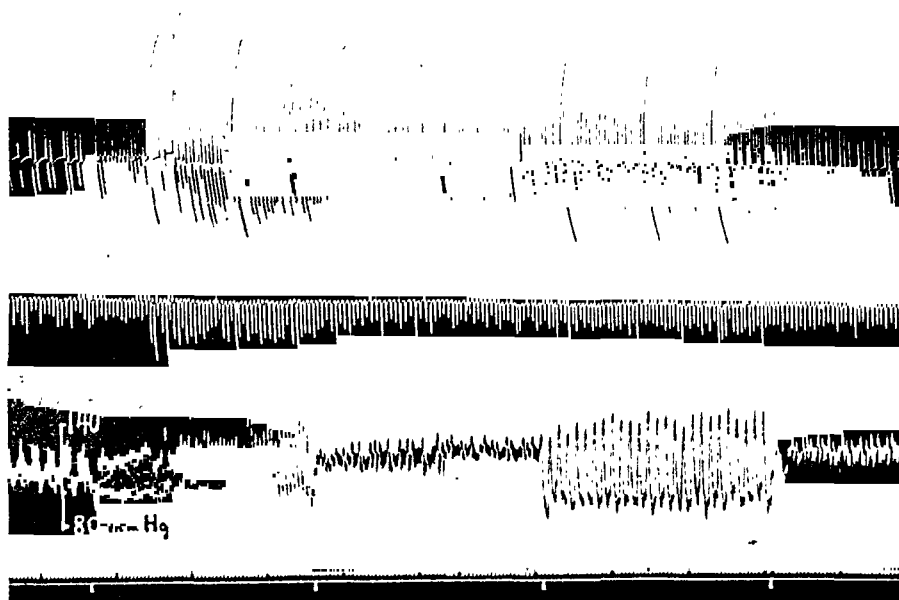


Fig. 4. Determinations 22 and 24 (Preparation IV). Donor dog (A) 18 kg., dog B 12 kg. Head of B perfused from one carotid artery and both external jugular veins of A. Curves represent from above respiration of A, respiratory movements of perfused head, blood pressure of A, time in minutes and periods of 3 seconds. Between marks electrical stimulation of A with modulated currents 20 times a minute (left part) and 8 times a minute (to the right). Cp. table 3.

had respired pure oxygen for several minutes. The results of three such determinations have been given in table 2 and should be compared with the three experiments immediately preceding them. It will be seen that the answer of the perfused head after oxygen is less pronounced during the period of work as well as after it. Fig. 5 gives the determinations 25 and 26. In this case the respirations of the donor dog were recorded. A rather close parallelism can be observed between the respiratory movements of the donor and the perfused head. The blood pressure also was somewhat influenced by the oxygen inhalation, the small rise in the systolic value during the contractions in air breathing being nearly absent when oxygen was given — another indication that some degree of oxygen want occurred during the work experiments performed during air breathing in comparison with the determinations during oxygen inhalation. The results clearly support the view that oxygen want is one of the factors that lead to increased ventilation during muscular work.

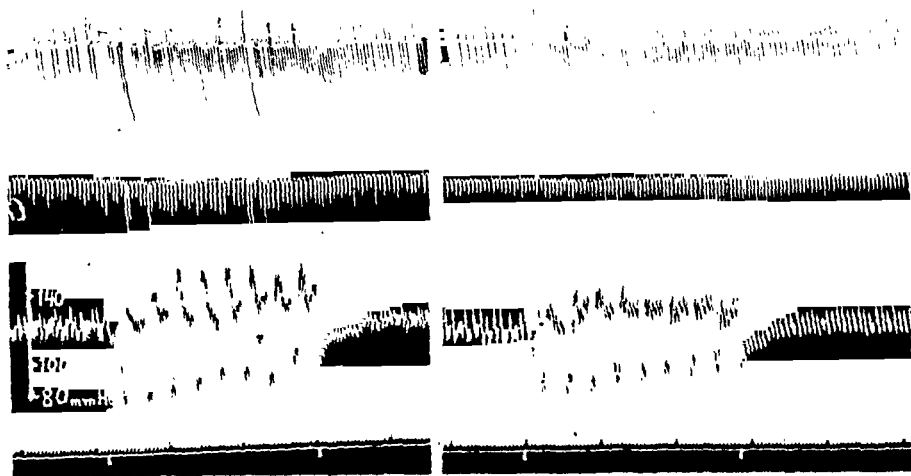


Fig. 5. Determinations 25 and 27 (Preparation IV). Designations as in fig. 4. Between marks electrical stimulation of dog A with tetanizing currents (15' + 5'') to the left when breathing air, to the right when breathing oxygen. Cp. table 2.

4. Influence of Chemoreceptor Denervation.

Since it is well known that oxygen want stimulates respiration by way of a reflex over the carotid and aortic bodies, whereas it depresses the respiratory centre, it seemed necessary to investigate the effects of muscular work on the perfused head after its carotid sinus mechanism had been denervated.

The immediate result of denervation in the three cases, where it was performed (I, III and IV), was a great reduction in the activity of the respiratory centre. In preparation I the respiratory movements became very shallow and the rate decreased from 12 to about 2 per minute. Carbon dioxide given to the donor dog still gave a good increase of the rate in the perfused head, however, so that muscular work could be tried. In preparation III the spontaneous rate of respiration after denervation sunk from 13.4 to 8.6 and in preparation IV from 17 to 8.8. These very considerable reductions show that the stimulation of the sinus mechanism is of great importance also during rest under the conditions studied a result which is in complete harmony with earlier observations from our laboratories.

Table 4.

Preparation	Number of determination	Duration of stimulation sec.	Period of stimulation min.	Respiratory movements of the perfused head after denervation of the carotid sinus mechanism, during stimulation with tetanizing currents				
				Rate per minute before work	Rate per minute during work	Amplitude during work	Rate per minute after work	Amplitude after work
I .	12	10+5	3	2	7	+	0	
II .	17	10+5	4	8.6	10.8	+	10.7	5
III .	18	5+5	4	12.0	12.0	+	12.5	(+)
IV .	27	15+5	3	8.8	12.2	++	0	

Table 4 gives the results after sinus denervation.

In all cases a good increase in the respiration of the perfused head had been induced during the work of the donor — for several reasons a quantitative comparison with the values obtained before denervation is difficult to perform —, but a great difference is now observed with regard to the behaviour after the working period. In determination 17 there was still an increase in rate after work has stopped, but the amplitude was not increased; in determination 18 a small increase in amplitude was seen at this stage. In determinations 12 and 27, however, respiration in the perfused head stopped altogether for a considerable time after the end of the work. It seems probable that this was due to a decrease in the carbon dioxide which was not compensated by oxygen want, which has only a depressing action on the centre. In determination 27 the dog began to awake from his anæsthesia — the experiment had lasted for several hours — and probably overventilated. This view is strengthened by the fact that about ten minutes later the dog was breathing very rapidly with typical Cheyne-Stokes periods. It could be expected if our interpretation was correct that it might be possible to induce some respiratory activity of the perfused head in this case by a new period of muscular work in the donor with a rise in the carbon dioxide tension. As shown in fig. 6 this supposition proved to be correct.

The results after denervation give clear evidence that the sinus mechanism as well as the centre is concerned with the increased respiration during muscular work. This conforms well with the results of EULER and LILJESTRAND quoted above.

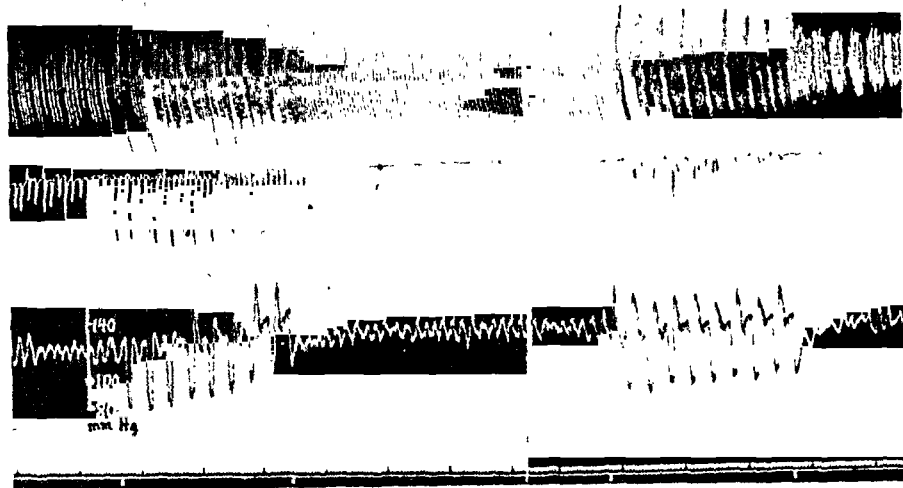


Fig. 6. Determination 27 (Preparation IV after sinus denervation of perfused head). Designations as in fig. 4. Between marks electrical stimulation of dog A with tetanizing currents (15'' + 5''). To the right same stimulation after prolonged apnea. Cp. table 4.

Discussion.

The determinations reported in this paper provide a clear demonstration that during and to some extent after very moderate muscular work, respiration is stimulated to increased activity through the mediation of the arterial blood. It must be emphasized that the conditions in our experiments were rather unfavourable to prove such an influence, since the electrical stimulation used not only produced muscular contractions but undoubtedly at the same strongly stimulated sensory nerves — certainly to a much greater degree than can be possible during actual work. This might well induce increased ventilation and mask the significance of the blood factor as demonstrated in our earliest experiments (table 1). Still the experiments performed leave no doubt at all about this influence through the blood. It seems probable that during muscular work the chemical alterations of the blood are of fundamental importance for the regulation of respiration.

Oxygen was found to decrease the respiratory response of the perfused head to muscular work. This indicates that part of the stimulation is caused by a certain degree of oxygen want. The fact

that after denervation of the carotid sinus the effects after the work are abolished speaks in the same direction.

Since overventilation greatly reduced the stimulating effect of the arterial blood on the respiration of the perfused head, it seems probable that part of the effect is due to increased carbon dioxide tension. This is also indicated by the fact that respiration was still stimulated by the perfusing blood after carotid sinus denervation. Since the centre does not react with increased activity for oxygen want — an experience that is supported by our results referred to under 4 — this effect is probably due to carbon dioxide (including other acids). It is interesting, however, that the stimulating effect of a moderate amount of carbon dioxide accumulation will over-compensate the depressing action of the corresponding oxygen want, so that the arterial blood from the working dog will still be able to stimulate the centre itself.

Summary.

Experiments have been performed in dogs on the reactions of the perfused isolated head to alterations in the perfusing blood provoked by muscular work of the donor dog.

It has been found that respiration of the perfused head is greatly stimulated during and often also after muscular work of the donor. Overventilation of the donor dog decreased but did not quite abolish the answer from the perfused head. The reaction could be decreased by oxygen inhalation of the donor. The after-effects when work was finished varied with the kind of work performed, being greater the more the static component of the work was pronounced. Carotid sinus denervation did not abolish the stimulating effect of the blood during muscular work, but the effect after work was decreased or even completely abolished.

The results are interpreted as giving evidence that during very moderate muscular work, oxygen want as well as increased carbon dioxide tension stimulate respiration in part reflexly over the carotid sinus mechanism, in part directly on the centre.

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The Formation Mechanism of Oestrogenic Hormones.¹

II. The Presence of the Oestrogen-Precursor in the Ovaries of Rats and Guinea-Pigs.

By

LENNART CLAEßSON and NILS-ÅKE HILLARP.

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Investigations into the biosynthesis of oestrogenic hormones in the ovary of rabbits have furnished evidence that this takes place on the basis of a precursor which is demonstrable by polarization optical and histochemical methods (CLAEßSON and HILLARP 1946, 1947, a). The precursor consists of a sterol of the cholesterol type, probably identical with cholesterol. It is stored in the interstitial gland and the theca interna. The amount of the oestrogen-precursor stored varies in conformity with the phases of the sexual cycle, viz. during oestrus, pregnancy and pseudo-pregnancy considerable amounts are present in the cells whilst in the immature or anoestrus ovary the precursor is either absent or it is present in scant quantities. These investigations have also shown that the precursor is mobilized in the ovary and disappears when the secretion of oestrogenic hormones is intensified, viz., at coitus or on injection of gonadotrophic hormone.

Before proceeding to the description of the investigations into the mechanism controlling the formation of the oestrogen-precursor and its transformation into hormone mention should be made of the variations in the precursor content of the ovaries of animals with an oestrus cycle, viz. rats and guinea-pigs.

¹ This study was aided by a grant from the Swedish Society for Medical Research.

Materials and Methods.

The specimens under study consisted of ovaries removed from albino rats and guinea-pigs during different phases of the oestrus cycle, during pregnancy and at different times after coitus (after coitus only rat ovaries were removed). The mobilization of the oestrogen-precursor from the ovary was also studied after administration of gonadotrophic hormone (PMS or PU). — The phases of the oestrus cycle in the rat were determined from vaginal smears whereas in the guinea-pig they were determined from the histological appearance of the vaginal epithelium. Animals with an irregular cycle were excluded.

Frozen sections (10 μ) of ovaries fixed in formalin were examined by polarization optical and histochemical analysis in accordance with the technique published in an earlier communication (CLAESSON and HILLARP, 1947, a). — The determinations of the variations in the precursor content were made in duplicate, one polarization optically and the other by Schultz' reaction. Applied to the ovaries of rabbits these methods yielded identical results, whereas applied to ovaries of rats and guinea-pigs divergent results were occasionally obtained. It was found that ovaries of rats and guinea-pigs must be fixed over a prolonged period or the precursor is not brought out in a visualizable form by polarized light. If the specimens were fixed for less than ten days, polarization optical analysis is undecisive, and the variations in the precursor content must then be determined on the basis of Schultz' reaction.

In the Tables appended to this paper + and +++++ stand for the approximate precursor content, +++++ indicating the maximum amount identified.

Results.

Rat.

1. *The Precursor Content during the Oestrus Cycle.*

The amount of oestrogen-precursor varied in accordance with the phases of the oestrus cycle though marked variations did not occur. In no phase of the cycle was the entire store of the precursor mobilized.

During pro-oestrus (Fig. 1) the precursor was stored in large quantities throughout the well developed interstitial gland (Table I). During the subsequent oestrus phase (Fig. 2) there was a noticeable fall in the precursor content, which was followed by a rise during met-oestrus (Fig. 3). Table I shows that the pictures during di-oestrus are not uniform. In the majority of the animals,

¹ Messrs. Leo A.-B., Hälsingborg, have kindly assisted us in our investigations by placing the gonadotrophic hormone preparations Antex and Physex Leo to our disposal.

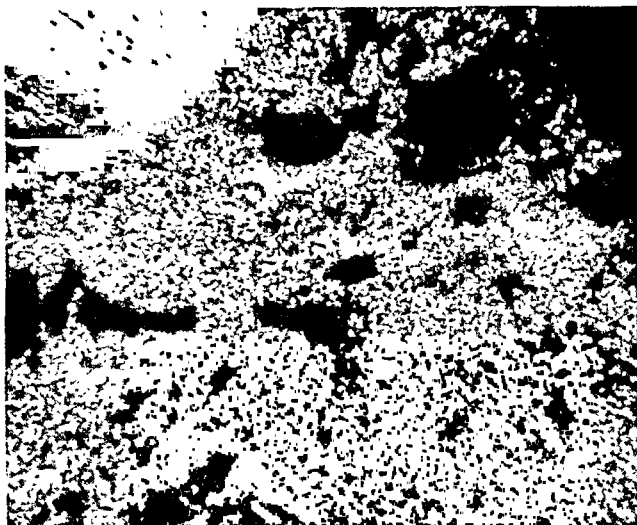


Fig. 1. Ovary from rat in pro-oestrus (No. 1, Table I). Large amounts of precursor stored throughout the interstitial gland. Frozen section (10μ) through cortical area. Crossed Nicols. $\times 80$.

however, the store of the oestrogen-precursor was comparatively small during this phase (fig. 4) as compared to the large amounts present during met-oestrus and pro-oestrus. The four animals (Nos. 18—21), whose pictures conform to pro-oestrus were killed at the time of the transitional stage between dioestrus and pro-

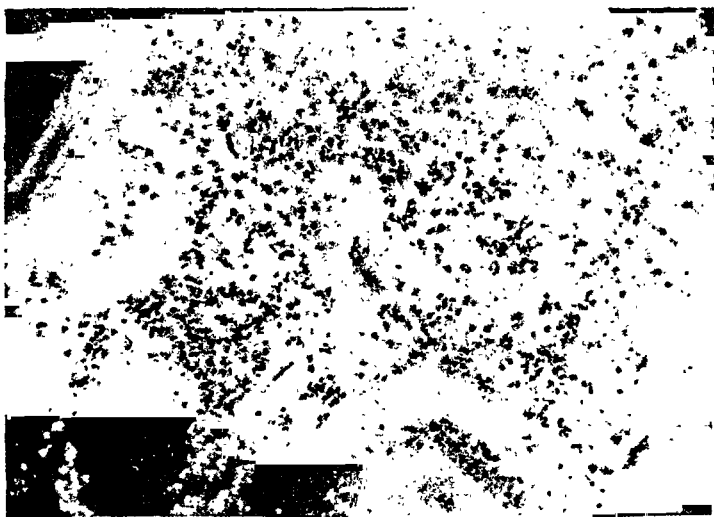


Fig. 2. Ovary from rat in oestrus (No. 14, Table I). Scarcity of precursor in the interstitial gland. Frozen section (10μ) through the cortex. Crossed Nicols. $\times 80$.

Table 1.

Precursor Content of the Theca Interna and Interstitial Gland of the Rat Ovary during Different Phases of the Oestrus Cycle.

1. Pro-oestrus			2. Oestrus		
Animal No	Interstitial Gland	Theca interna Preovulatory Follicles	Animal No	Interstitial Gland	Theca interna Preovulatory or recently ruptured Follicles
1	+++ and ++++	++	1	++	+
2	+++		2	+++	0 and +
3	++ and ++++		3	++	<+
4	+++ and ++++	+++	4	++	0 and +
5	++ and ++++		5	++	+
6	+++ and ++++	+++	6	++	0 and +
7	+++		7	++	<+
8	++ and ++++	+++	8	++	0
9	+++	+++	9	++	0
10	+++ and ++++	+++	10	++	0 and +
11	+++	+++	11	++ and ++++	0 and +
12	+++	+++	12	++ and ++++	0 and +
			13	++ and ++++	0 and +
4. Dioestrus					
		Theca interna Graafian Foll.			
1	++ and ++++	0	14	+ and ++	0
2	+	0	15	+ and ++	0 and +
3	+ and ++	+	16	++ and ++++	+
4	++ and ++++	+	17	++ and ++++	0
5	++	0	18	++ and ++++	0 and +
6	++	0	19	+ and ++	0 and +
7	++	<+	20	+ and ++	0
8	++	0 and +	21	++	+
9	+++	0 and +	22	++ and ++++	<+
10	++ and ++++	0 and +	23	++	0 and +
11	+++	<+	3. Metoestrus		
12	++ and ++++	+ and ++			Theca interna of Graafian Foll.
13	++ and ++++	0 and +			
14	++ and ++++	0			
15	++	0 to ++++	1	+++ and ++++	0
16	+	0 and +	2	+++ and ++++	0 and +
17	++ and ++++	0 and +	3	+++ and ++++	0
18	++++	++ and ++++			
19	++++	0 and +			
20	+++	0 and +			
21	++++	0 and +			

oestrus, which would explain the large amounts of the precursor present.

The cells of the theca interna likewise showed characteristic variations in the precursor content during the oestrus cycle. During pro-oestrus profuse amounts were present only in the large

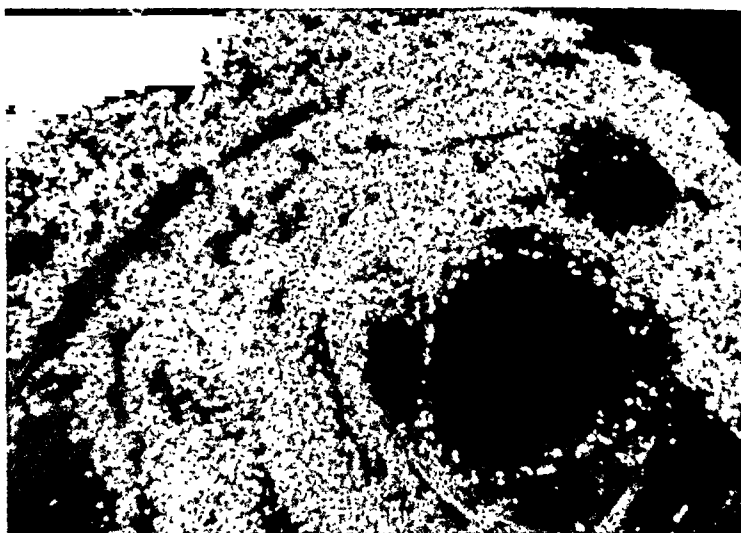


Fig. 3. Ovary from rat in metoestrus (No. 2, Table I). Large amounts of precursor stored in the interstitial gland. Frozen section ($10\ \mu$) through the cortex. Crossed Nicols. $\times 80$.

preovulatory follicles (Table 1) which appear in sections in the form of a ring encircling the follicles (Fig. 5). At ovulation the precursor was mobilized from the theca interna cells, which was proved by the fact that it was then not visualizable in the theca cells around recently ruptured follicles (Fig. 6). On the other hand,

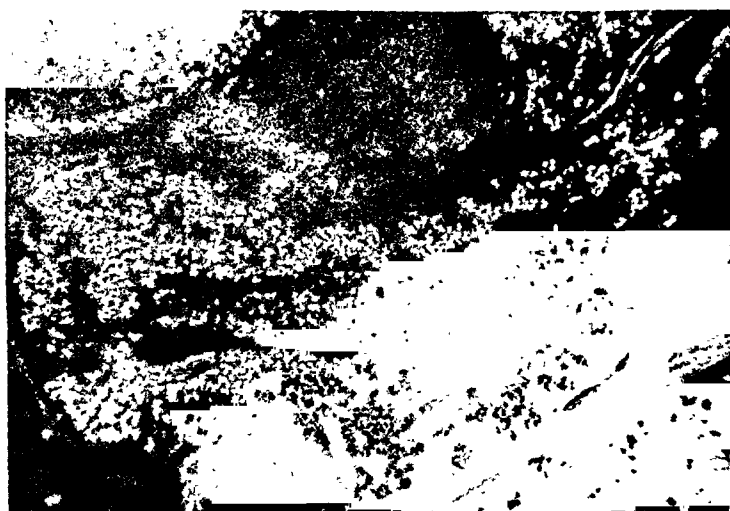


Fig. 4. Ovary from rat in dioestrus (No. 15, Table I). Scarcity of precursor in the interstitial gland. Frozen section ($10\ \mu$) through the cortex. Crossed Nicols. $\times 80$.

in the theca of the growing follicles there were no noticeable variations in the precursor content. During all the phases of the oestrus cycle the precursor was either absent or it was present in negligible quantities.

Hence, the precursor of oestrogenic hormones in the interstitial gland of the rat ovary shows two marked peaks during the oestrus

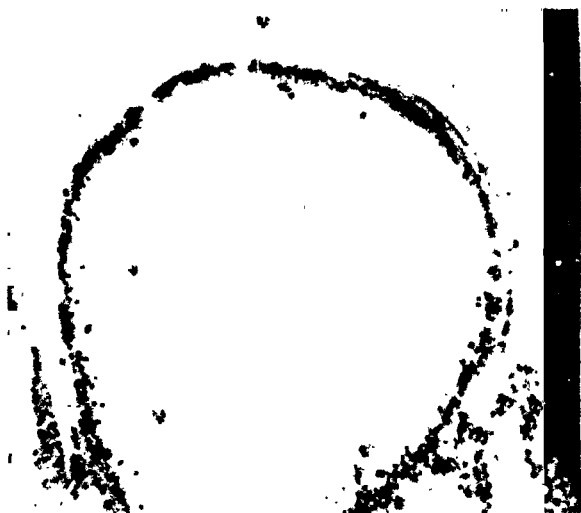


Fig. 5. Preovulatory follicle from ovary removed during pro-oestrus (rat No. 11, Table I). Large amounts of precursor stored in the cells of the theca interna. Frozen section ($10\ \mu$) through the cortex. Crossed Nicols. $\times 80$.

cycle, viz. during pro-oestrus and met-oestrus. During the intermediate phases, viz. oestrus and dioestrus the precursor content of this gland is very low. In the cells of the theca interna large quantities are deposited solely in the preovulatory follicles. At the time of ovulation the oestrogen-precursor is here mobilized and disappears.

2. The Precursor Content post Coitum.

The precursor content was determined in the ovaries of animals killed post coitum at different times between three and a half and twenty-four hours counting from the beginning of copulation. Only those female animals were included in the study, in whose vaginas sperms were demonstrable one hour after they had been mated.

Already after three and a half hours the precursor content of the interstitial gland had considerably decreased (Table II). In the majority of the glandular lobes the precursor was not traceable whereas the remaining ones responded only weakly to Schultz'

reaction. This low precursor content was maintained for fifteen hours post coitum. In one (No. 8) of the two animals, which were killed twenty-four hours post coitum, it was found to be higher as compared to that determined in the other animals under study. This might be explained by assuming that the storing of the precursor, which leads to the massive accumulation characteriz-

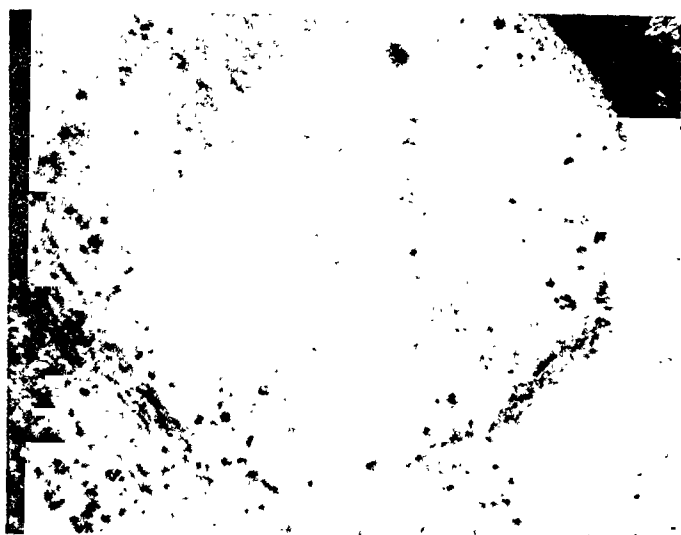


Fig. 6. Recently ruptured follicle from ovary removed during oestrus (rat No. 12, Table I). Absence of precursor in the theca interna. Frozen section ($10\ \mu$) through the cortex. Crossed Nicols. $\times 80$.

ing the ovary of the pregnant animal (see statements under point 3) begins at this time.

In interpreting the picture of the oestrogen-precursor in the interstitial gland post coitum, the fact must be taken into consideration that there is normally a marked fall in the precursor content during the intermediate stage between pro-oestrus and oestrus. If, however, Tables I and II are compared, it will become evident that post coitum it is considerably lower than at the time of oestrus. It would thus appear that in rats coitus causes a fall in the precursor content of the interstitial gland.

Three and a half hours and five hours post coitum there was no evidence of the precursor in the theca interna of the preovulatory follicles. It was likewise absent in the theca cells of recently ruptured follicles, which were about to be transformed into corpora lutea. Since, however, under normal conditions the oestrogen-precursor is mobilized in this structure at the time of ovula-

Table II.

Precursor Content of the Theca Interna and Interstitial Gland of the Rat Ovary at Different Times post coitum.

Animal No	Period post coitum (hours)	Interstitial Gland	Theca Interna
1	3½	0 and +	0 Preovulatory follicles
2	5	0 and +	0 " " "
3	5	0 and +	0 " " "
4	10	+	0 Recently ruptured follicles
5	12	0 and +	0 " " "
6	15	0 and +	0 " " "
7	24	0 and +	0 Recently formed corpus luteum
8	24	+ and ++	0 Recently ruptured follicles

tion, it is not possible definitely to state whether coitus intensifies this process.

Coitus thus causes a marked fall in the oestrogen-precursor content of the interstitial gland within a short period of time.

3. The Precursor Content during Pregnancy.

The ovaries under study were removed from pregnant animals at various times (between the third and twenty-first day) during pregnancy. As may be seen from Table III, the precursor content of the interstitial gland shows some variations. These variations, however, do not seem to be significative of the fact that the oestrogen-precursor content at a certain stage of pregnancy would differ from that present at the other stages. Generally speaking, large amounts were present throughout pregnancy (fig. 7). On the basis of the specimens studied it cannot be definitely stated, whether the precursor content changes significantly immediately before partus. These conditions will be investigated in a special study.

In the theca interna of the graafian follicles large amounts were present till the fifteenth day of pregnancy. On the other hand, from that day until the twentieth day of pregnancy the precursor was absent in the theca cells, whereas on the twentieth and twenty-first day there was again evidence of its storing. — In the theca cells of the small follicles it was either absent or only small amounts were present, conditions, which conform to those identified during the oestrus cycle.

Table III.

Precursor Content of the Interstitial Gland and Theca Interna of Ovaries removed from Pregnant Rats, Untreated or Treated with Gonadotrophic Hormone. (Simultaneously with the injection of the hormone one ovary (R) was extirpated; the other (L) was removed 19—48 hours after the injection.)

Animal No	Day of Pregnancy	Dosis of Gonadotrophic Hormone subcutaneously injected	Number of Hours between Extirpation of the two Ovaries	Interstitial Gland	Theca Interna Graafian Follicles
1	2—3			++ and+++	+
2	2—4			+++	+++
3	5	50 I.U. Physex	48	R +++ and++++ L 0	++ 0
4	5			+++	++
5	5	35 M. U. Antex	48	R +++ L 0	++ 0
6	5	35 M. U. Antex	48	R +++ L 0	++ 0
7	5	50 I. U. Physex	48	R +++ and++++ L 0	+++ 0
8	8	15 M. U. Antex	23	R +++ L ++	+++ ++
9	8	35 M. U. Antex	28	R ++++ L + to ++++	+++ +
10	8	50 I. U. Physex	48	R ++ and++++ L 0	+++ 0
11 Right	8			+++	+++
12 " "	8			+++	+++
13	9	15 M. U. Antex	23	R ++++ L +++	+++ +++
14	10	35 M. U. Antex	28	R ++++ L +	+++ +
11 Left	10			+++	+++
12 " "	10			+++	+++
15	11	10 M. U. Antex	19	R +++ L +++	+++ +++
16	15	10 M. U. Antex	19	R ++++ L ++++	+++ +++
17	15	15 M. U. Antex	19	R ++++ L ++++	+++ +++
18	16	35 M. U. Antex	48	R ++++ L 0	0 0
19	16	35 M. U. Antex	32	R ++++ L ++	0 0
20	19			++++	0
21	19	35 M. U. Antex	28	R +++ and++++ L + and++	0 0
22	20			++ and++++	0
23	20			++++	++
24	20			++ and++++	++
25	21			+ to +++	+++
26	21			+++	++

Hence, the oestrogen-precursor content of the interstitial gland is maintained at a high level throughout pregnancy. Until the sixteenth day of pregnancy this applies also to the theca interna of the graafian follicles. Between the sixteenth and twentieth day, however, the precursor is absent in the theca cells, whereas in the days preceding partus there is again evidence of accumulation.

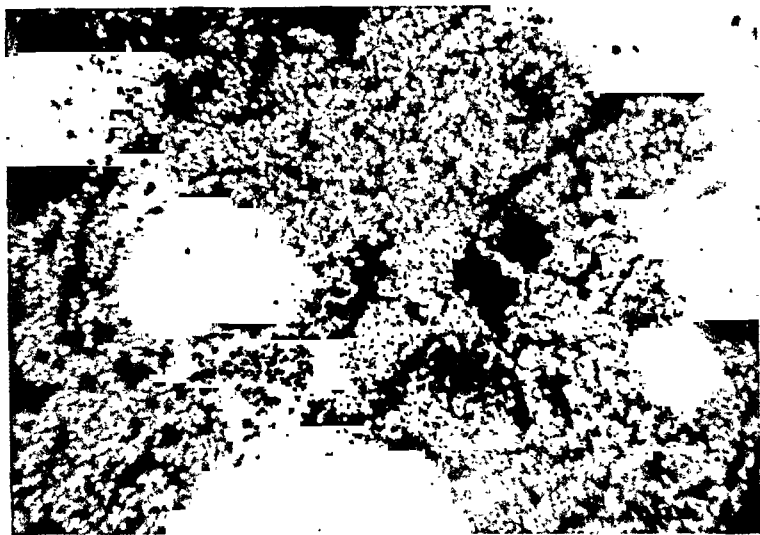


Fig. 7. Rat ovary (right) removed on the 16th day of pregnancy (No. 18, Table III). Abundance of precursor in the interstitial gland. Frozen section ($10\ \mu$) through the cortex and medulla. Crossed Nicols. $\times 80$.

4. The Precursor Content of Gonadotrophic-stimulated Ovaries.

The rapid changes in the precursor content during the oestrus cycle made it necessary to investigate the effect of gonadotrophic stimulation on ovaries in pregnant animals. In order to make it possible to compare the precursor content in one and the same animal before and after gonadotrophic stimulation (Table III), one ovary was extirpated immediately before injecting the hormone. The ovary, which was primarily left, was later on removed at different times after the injection, but not later than after forty-eight hours. The animals Nos. 11 and 12, which were not treated with gonadotrophic hormone furnish evidence of the fact that the removal of one ovary does not noticeably change the precursor content of the other ovary left primarily in situ.

Table III shows that 35 mouse units of PMS or 50 international units of PU subcutaneously injected causes mobilization of the precursor from the ovary after fourthy-eight hours. In all animals

treated in this manner the precursor was mobilized and disappeared in both the interstitial gland and the theca interna. Neither polarization optically or by Schultz's reaction was there any trace of the precursor demonstrable (Figs. 7 and 8). On the administration of weaker doses of gonadotrophic hormone, however, though causing to a certain extent its mobilization from these structure, smaller quantities were left.

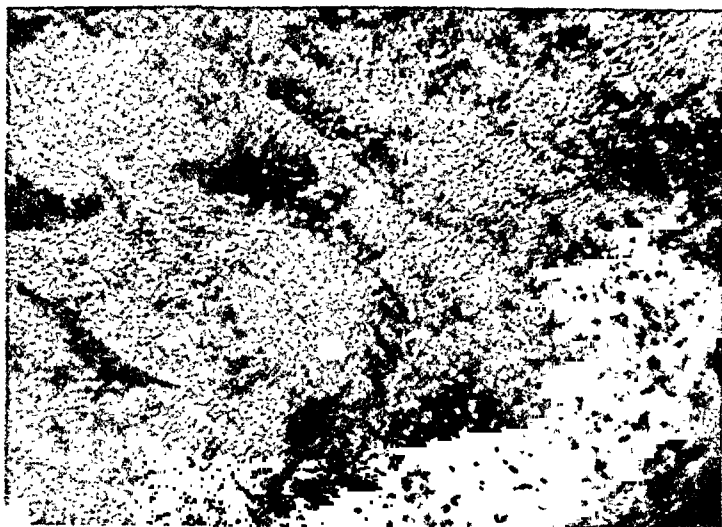


Fig. 8. Left ovary from the same animal as the ovary shown in Fig. 7, removed 48 hours after injection of 35 mouse units of pregnant mare serum gonadotrophins. Absence of precursor in the interstitial gland. Frozen section ($10\ \mu$) through the cortex. Crossed Nicols. $\times 80$.

After the administration of large doses of gonadotrophic hormone to pregnant animals the oestrogen-precursor is mobilized and disappears completely in both the theca interna and the interstitial gland.

Guinea-pig.

1. *The Precursor Content during the Oestrus Cycle.*

The oestrogen-precursor content of the interstitial gland varied also in the ovary of the guinea-pig though to a lesser degree than in the ovary of the rat because there was no extensive accumulation during the phases of the oestrus cycle (Table IV). In this gland it reached its peak during the long period of di-oestrus though it happened occasionally that it was low at an early stage of this phase. In contrast to the conditions observed in rats, there

Table IV.

Precursor Content in the Interstitial Gland of the Guinea-pig Ovary during different Phases of the Oestrus Cycle. (The first day of the cycle coincides with the day when the vagina opens.)

1. Pro-oestrus		3. Di-oestrus		
Animal No	Interstitial Gland	Animal No	Day of Cycle	Interstitial Gland
1	++	1	2	+
2	+	2	2	+++
3	+	3	3	++
4	++	4	3	+++
5	++	5	4	++
6	++	6	4	0 and +
7	++	7	4	+++
8	+	8	4	+++
9	+++	9	5	++
10	++	10	6	+++
11	+ and ++	11	7	++
12	0 and +	12	8	+++
2. Oestrus		13	9	++
1	<+	14	10	++ and +++
2	++	15	11	++
3	+	16	12	+++
4	+	17	12	+++
5	+	18	13	++
6	++	19	13	+++
7	++	20	14	+++
8	+			
9	++			
10	+			
11	+ and ++			
12	<+			
13	0 to ++			
14	0 and +			
15	0 and +			
16	++			
17	+			

was no marked precursor accumulation during pro-oestrus; on the contrary, there was a fall in the precursor content rather. This fall was still more marked during oestrus, the content being then lowest as compared to the other phases of the cycle.

In contrast to the conditions in the rat, the precursor was either absent in the cells of the theca interna or it was present in such small quantities that it was hardly demonstrable. This applies to both growing and preovulatory follicles.

Hence, in the interstitial gland of the ovary of the guinea-pig the precursor is demonstrable during all the phases of the cycle though

Table V.

Precursor Content of the Interstitial Gland of Ovaries removed from Pregnant Guinea-pigs.

Animal No	Day of Pregnancy	Interstitial Gland
1	4	< +
2	8	+
3	12	+
4	16	< +
5	20	++
6	24	+
7	28	+
8	32	++
9	36	+ and +++
10	40	++ and +++
11	44	++ and +++
12	50	++ and +++
13	50	++ and +++
14	60	++ and +++

comparatively inconsiderable amounts are stored. The precursor content reaches its peak during dioestrus, being lowest at the time of oestrus. In the theca cells there is either no evidence of the precursor or it is present in such inconsiderable quantities that it is hardly demonstrable.

2. The Precursor Content during Pregnancy.

The ovaries under study were removed from pregnant animals at different stages of pregnancy, *i. e.* between the fourth and sixtieth day of pregnancy. Throughout the preceding period till about the fourtieth day of pregnancy the precursor content was very low in the interstitial gland (Table V). From then on the pictures changed considerably. Until the sixtieth day of pregnancy large quantities of precursor were stored in the interstitial gland though to a lesser degree as compared to those deposited in the rat.

In the theca interna there was no evidence of the precursor during pregnancy.

Hence, the precursor content of the interstitial gland of the guinea-pig ovary is very low during the first two thirds of pregnancy whereas during the last third there is a marked accumulation in the cells of this gland.

Discussion.

In earlier communications (CLAESSON and HILLARP, 1946, 1947, a and b) the present authors demonstrated the presence of a sterol in the interstitial gland of the rabbit, rat and guinea-pig ovaries. Polarization optical and histochemical analyses suggested that the sterol was identical with cholesterol. In the rabbit it was possible to furnish direct evidence that it was engaged in the hormone metabolism in the form of a precursor of oestrogenic substances. Indirectly it was thus proved that it played a part also in the ovaries of the rat, and guinea-pig. The investigations discussed in this paper, particularly those into the mobilization of sterol from the gonadotrophic-stimulated ovary, have furnished direct evidence in support of the assumption that the sterol is the precursor of oestrogens also in interstitial gland of the rat and guinea-pig.

In a series of papers DEMPSEY and BASSETT (1943), DEMPSEY and WISLOCKI (1944, 1946), WISLOCKI and DEMPSEY (1946), published their investigations into the steroid formation in the ovary, testis and placenta. They used histochemical reactions which they assumed to be indicative of steroid substances. The results of their investigations led them to the assumption that these structures contained keto-steroids, which were histochemically demonstrable. The present study furnished evidence for the formation of hormones in the ovary, which is quite contrary to the hypothesis of these authors. *From both the chemical and biological points of view the methods of examination used in our studies brought forward evidence in support of the assumption that the formation of oestrogenic hormones is based on a sterol of the cholesterol type, which is engaged in the formation mechanism as a precursor of the active hormone.* The histochemical reactions for keto-steroids, which were used by DEMPSEY, BASSETT and WISLOCKI were critically studied in a preceding paper (CLAESSON and HILLARP, 1947, b) DEMPSEY, BASSETT and WISLOCKI based their methods of study on the assumption that these reactions are due to one and the same substance. The above mentioned investigations, however, have demonstrated that this is erroneous, and therefore their hypothesis cannot be accepted.

The precursor of oestrogenic hormones having been demonstrated it is within the range of possibility to investigate what structures

are responsible for the production of these hormones. As early as in 1917 (STEINACH, 1917, STEINACH and HOLZKNECHT, 1917) the view was advanced that the interstitial gland of the ovary would be the site for the formation of oestrogenic hormones. On the basis of the results of histological investigations into the interstitial cells (STIEVE, 1921, KINGSBURY, 1914, 1939, SCHRÖDER, 1930) and of those obtained with extraction and implantation experiments (ALLEN and DOISY, 1923, 1924, ALLEN, PRATT and DOISY, 1925, ZONDEK and ASCHHEIM, 1926, ASCHHEIM 1926, ALLEN, PRATT, NEWELL and BLAND, 1930), however, this concept was largely modified. The last mentioned authors believed that the walls of the follicles were the source of the oestrogenic substances. Other authors, who made similar experiments, felt that this was not true (DICKENS, DODDS and WRIGHT, 1925, PARKS and BELLERBY, 1926). The results of experimental X-ray irradiation of the ovaries seemed likewise to support the assumption that the follicles were not of fundamental importance in the formation of the hormones (PARKES, 1926, 1927, 1928, BRAMBELL and PARKES, 1927, BRAMBELL, PARKES and FIELDING, 1927, GELLER, 1929—30, GENTHER SCHMIDT, 1936). WESTMAN (1930), however furnished evidence that this conclusion was ill founded because the origin of the hormone-producing cells after X-ray irradiation of the ovary was identical with that of the follicles. CORNER (1938), ALLEN, HISAW and GARDNER (1939), and ALLEN (1941) on the basis of their investigations into the ovary and its endocrine functions supported the assumption that the walls of the follicles would be the site for the formation of oestrogenic substances. Against this background one might be led to assume that the interstitial gland situated outside the theca interna would not be concerned in the formation of oestrogenic hormones. Since it has hitherto not been demonstrated that this ovarian component, which is the dominating element of the ovary in the rabbit and other animals, is concerned in the formation of progesterone, the significance of this gland would be obscure. The present authors, however, have forwarded evidence that from a quantitative point of view the interstitial gland is the main site of storing of the oestrogen-precursor. Moreover, in rats and guinea-pigs the oestrogen-precursor content shows characteristic variations during the normal oestrus cycle, and in rabbits and rats the oestrogen-precursor is mobilized within a few hours after coitus. *These facts are convincing evidence that the interstitial gland is of fundamental*

importance in the normal formation of oestrogenic substances in rabbits, rats and guinea-pigs.

In rabbits and rats the oestrogen-precursor has also been demonstrated in the cells of the theca interna. The part played by the latter in the formation of hormones cannot be deduced from the specimens under study. There are indications, however, that it functions independently at the side of the interstitial gland. The fact that the precursor disappears in the rat during a certain period of pregnancy (between the sixteenth and twentieth day) whereas the level of the precursor content of the interstitial gland is maintained during this period supports this assumption. The fact that there generally is no evidence of the precursor in the theca interna of the guinea-pig ovary (though occasionally it may be present but only in negligible quantities), neither during the oestrus cycle or during pregnancy, is further evidence in support of this view. The difference between the reaction of the theca interna and that of the interstitial gland seems to support the view advanced by WESTMAN (1934). The cytological investigations made by MOSSMAN (1937) and STAFFORD, COLLINS and MOSSMAN (1942) also suggest that the theca interna is concerned in the formation of hormones.

Since the factors, which have a bearing on the mechanism controlling the formation of the precursor and its transformation into active hormone are in the main still obscure it is in the present state of our knowledge not possible to explain the significance of the variations in the precursor content during the oestrus cycle and pregnancy. It is, however, worthy of emphasis, that there is a marked fall in the precursor content of the interstitial gland during the oestrus phase in both rats and guinea-pigs. It should here also be mentioned that there is a considerable difference between the precursor content of the ovaries of guinea-pigs and that of the ovaries of rats during pregnancy. In rats there is massive accumulation throughout pregnancy whereas in guinea-pigs the oestrogen-precursor is not stored before the end of pregnancy.

Summary.

The present investigation into the oestrogen-precursor in the ovaries of the rat and guinea-pig, as representatives of animals with an oestrus cycle, is a sequel to an earlier study on this precursor in the rabbit ovary.

The precursor content of the interstitial gland of the rat ovary shows two marked peaks during the oestrus cycle, viz. during pro-oestrus and met-oestrus. During the intermediate phases, *i. e.* oestrus and dioestrus, it is comparatively low. In the theca interna the precursor is stored in large amounts only in the pre-ovulatory follicles. At the time of ovulation this store is mobilized. In the interstitial gland of the guinea-pig ovary the oestrogen-precursor reaches its maximum during dioestrus, and its minimum during oestrus; in the cells of the theca interna the precursor is either absent or it is present in such small amounts that it is hardly appreciable.

The precursor-content of the interstitial gland of the ovary of the pregnant rat is high. This high level is maintained unaltered throughout pregnancy. This applies also to the theca interna of the graafian follicles until the sixteenth day of pregnancy. Between the sixteenth and twentieth day, however, the precursor is absent in the theca cells whereas during the days preceding partus it is again stored.

In pregnant guinea-pigs the massive accumulation, which is present in the interstitial gland of the rat throughout pregnancy, does not take place until towards the end of pregnancy; in the theca interna the oestrogen-precursor is absent.

In rats, coitus causes over a short period of time a marked fall in the oestrogen-precursor content of the interstitial gland. On large doses of gonadotrophic hormone PMS or PU the precursor is mobilized and disappears from both the theca interna and the interstitial gland of the ovary of the pregnant rat.

The part played by the interstitial gland and the theca interna in the formation of oestrogenic substances in the ovary is discussed. The results of this study support the assumption that the interstitial gland is of fundamental importance in the normal formation of oestrogens in the rabbit, rat and guinea-pig.

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Further Studies of the Pulmonary Arterial Blood Pressure.

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The effects of inhalation of gas mixtures with varying oxygen and carbon dioxide content on the pulmonary arterial blood pressure of the cat have recently been studied by EULER and LILJESTRAND (1946). It was found that pure oxygen quickly led to a decrease in the pressure, whereas a rise was observed during oxygen lack. Carbon dioxide (6.5—20 per cent) in oxygen as compared with oxygen alone caused a small rise in the pressure. These results were interpreted as the expressions of an hitherto unknown regulating mechanism for an adequate distribution of the blood through the various parts of the lungs according to the efficiency of aeration.

In order to contribute to the analysis of this mechanism it seemed desirable to investigate whether drugs with a blocking effect on the sympathetic and parasympathetic systems would influence the variations in the pulmonary arterial pressure caused by alterations in the alveolar concentrations of oxygen and carbon dioxide. The present study was undertaken mainly to elucidate this point. The agents used for this purpose were ergotamine tartrate, dihydroergotamine tartrate, yohimbine hydrochloride and atropine sulfate. It was also investigated whether the effects mentioned were in any way influenced from the carotid sinus region. Incidentally the direct action on the pulmonary arterial

pressure of the drugs used was observed. Lastly studies were performed with regard to the effect of alterations in the ventilation and the respiratory resistance on the pulmonary arterial pressure.

Methods.

The experiments were made on cats anaesthetized with 0.06 g chloralose per kg intravenously. The systemic blood pressure was recorded with a mercury manometer from the femoral artery, the pulmonary arterial pressure according to the method described in detail by EULER and LILJESTRAND. When the cannula was introduced into the pulmonary artery, the vessel was clamped off between two thick threads instead of the pair of forceps used by the authors mentioned. One thread close to the heart was fixed on a "Ligaturstab", whereas the other was held by the operator. This had the advantage that the pulmonary artery could be lifted and fixed during the manipulations. In the earlier experiments the thorax was opened by a longitudinal incision in the middle of the sternum, the walls then being held apart by a self-retaining retractor. The thymus was dissected upward from the base of the heart and held back by a pair of haemostats in which case very little bleeding if any occurred. In experiments performed in this way a steady and continuous fall of the systemic blood pressure usually occurred till the death of the animal, probably due to loss of carbon dioxide through the exposed surface of the lungs. In later experiments another technique was therefore adopted. An incision was made on the left side of the thorax in the 4th intercostal space and the pulmonary artery exposed. Another smaller incision in the 5th intercostal space on the right side served to introduce and fix the "Ligaturstab". In successful experiments the cannula could be introduced in about 30 seconds after the circulation had been shut off. The thorax was then closed at an inflated state of the lungs. With this technique the systemic pressure could easily be kept within the normal range even for 2—4 hours after the experiment had been started.

Often the pressure was also recorded from the left auricle, a common venous cannula being inserted into the auricular appendage and connected to a glass tube and a piston recorder in the same way as described by EULER and LILJESTRAND for the registration of the pulmonary arterial pressure. When two cannulas were used in this way, the spontaneous respiration of the animal was often more or less impaired.

Gas mixtures kept in bags were administered either through the Starling pump in the cases of artificial respiration or attached to the inspiratory Müller valve when the animal breathed spontaneously. Changes in the degree of artificial ventilation for comparative purposes were effected by introducing a narrow glass tube into the expiratory outlet of the tracheal cannula during a certain period of time. Occlusion of the carotids was performed by means of small artery clips.

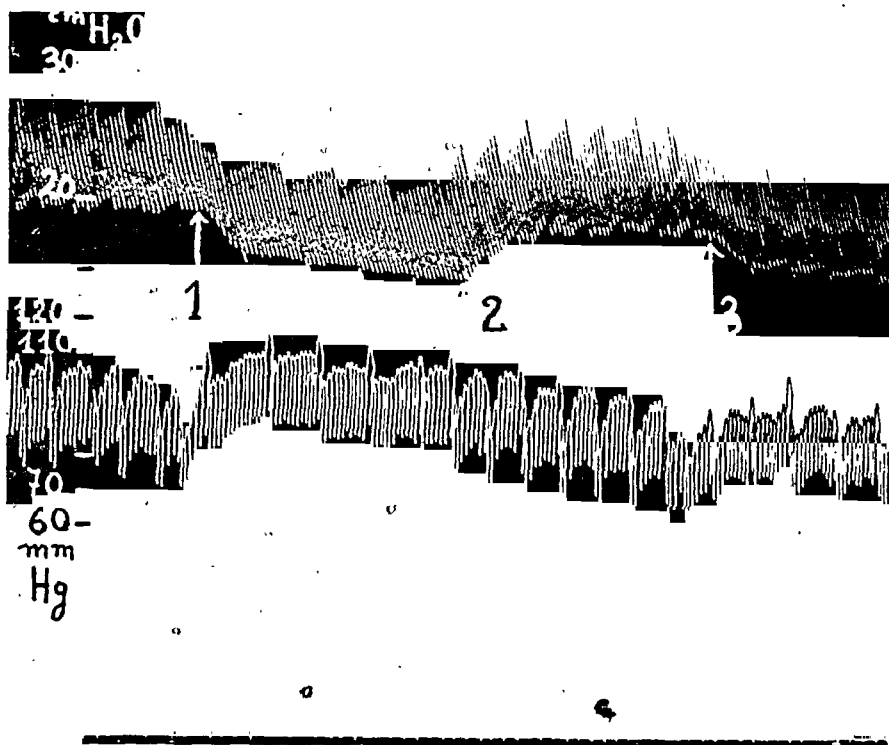


Fig. 1. Cat. 3.7 kg. Chloralose. Upper curve pulmonary arterial pressure, lower curve systemic blood pressure. 1. O_2 (from air). 2. Air. 3. Oxygen 50 p. c. in N_2 . Spontaneous breathing, closed thorax. Time 10 sec.

Results.

1. Effects of Inhalation of Gas Mixtures of Varying Oxygen and Carbon Dioxide Content.

In complete accord with the results obtained by EULER and LILJESTRAND, inhalation of pure oxygen quite regularly led to a distinct fall of the pulmonary arterial blood pressure. The fall was less pronounced after 50 per cent than after pure oxygen (fig. 1). Thus in the experiment quoted in fig. 1 pure oxygen decreased the pressure from 23 to 19 cm, whereas the corresponding values for 50 per cent oxygen were 21 and 19 respectively. In a second experiment a drop from 23.5 to 18.5 cm for pure but only from 21 to 18 for 50 percent oxygen was observed. During the early stage of inhalation of a gas mixture rich in oxygen a small rise (about 1 cm) of the pressure in the left auricle was found,

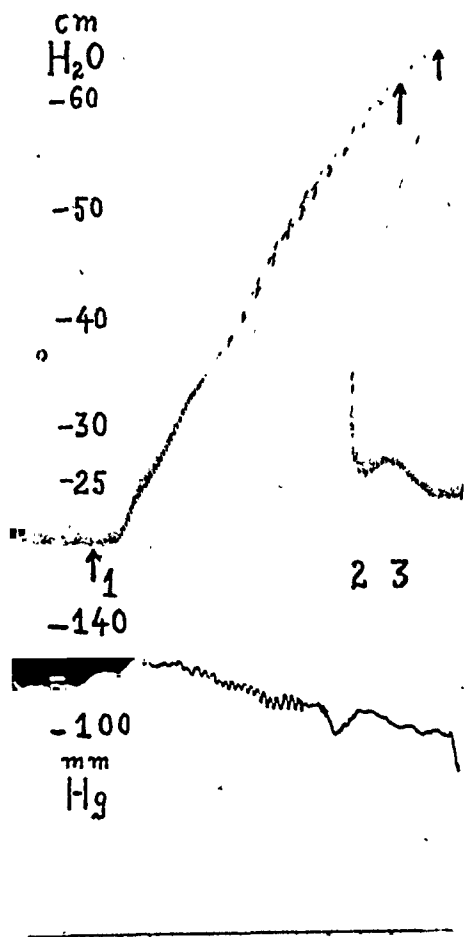


Fig. 2 A. Cat. 2.3 kg. Chloralose. Upper curve pulmonary arterial pressure, lower curve systemic blood pressure. 1. 15 p. c. O₂ in N₂ (from air). 2. Air. 3. O₂. Artificial respiration. Time 30 sec.

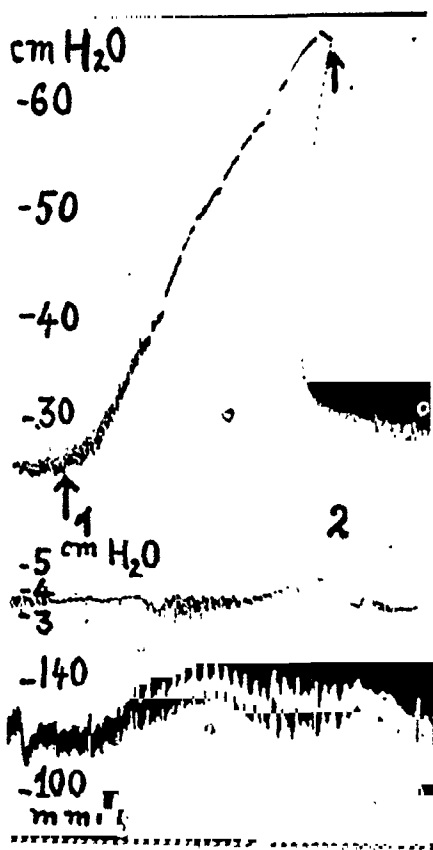


Fig. 2 B. Cat. 3.8 kg. Chloralose. Upper curve pulmonary blood pressure, middle curve left auricular pressure, lower curve systemic blood pressure. 1. Air (from O₂). 2. C₂. Spontaneous breathing. Time 10 sec.

presumably caused by a sudden increase of the inflow of blood when the vessels to the lungs became dilated.

Transition from air to a gas mixture of a lower oxygen content rapidly induced a rise in the pulmonary arterial pressure. Individual variations with regard to the sensitivity of the animal for oxygen and oxygen want were quite obvious. Fig. 2 A gives an illustration of a very big rise in the pressure caused by a reduction of the oxygen content from air to 15 per cent oxygen in

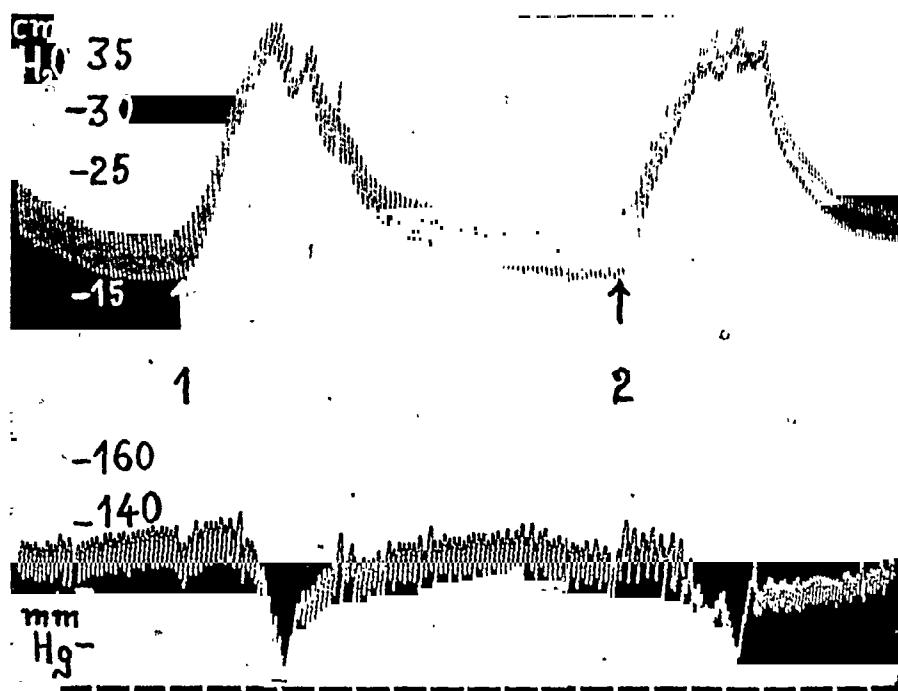


Fig. 3. Cat. 3.4 kg. Upper curve pulmonary arterial pressure, lower curve systemic blood pressure. 1. Interposing a rubber tube with a column of soda lime for one minute. 2. Interposing the same tube with a column of sand (the size of soda lime particles). Spontaneous breathing. Time 30 sec.

nitrogen. As a matter of fact the rise was so enormous that it was necessary to discontinue the inhalation of the gas mixture with the reduced oxygen content and shift to air and later on to oxygen. Of course it is possible that in this case the rise in the pulmonary arterial pressure might to some extent have been due to back pressure from the left auricle if the function of the heart had been impaired. From fig. 2 B, however, it can be seen that a very great effect on the pulmonary arterial pressure may be obtained from oxygen want without any impairment of the function of the heart. In this case even the shift from oxygen to air led to a rise in the pulmonary arterial pressure from 25 to 65 cm — possibly the ventilation was somewhat insufficient. The interesting point is that the pressure in the left auricle was hardly affected at all.

Substitution of 7 or 14 per cent carbon dioxide in oxygen for pure oxygen either had no effect at all on the pulmonary arterial pressure or led to a rather insignificant increase. It was therefore to be assumed that when the respiratory air is simultaneously



Fig. 4. Cat. 3.4 kg. Chloralose. Upper curve pulmonary arterial pressure, lower, curve systemic blood pressure. Time 30 sec. 1. O_2 (from air). 2. 7 p. c. CO_2 in O_2 . 3. Injection of 0.1 mg per kg ergotamine tartrate. 4. O_2 . 5. Air. 6. O_2 . 7. 7 p. c. CO_2 in O_2 . Arrows at lower curve indicate occlusion and opening of common carotid arteries. Spontaneous breathing.

enriched in carbon dioxide and reduced in oxygen content the effect on the pulmonary arterial pressure will almost completely be due to the latter factor. That such is the case, is clearly illustrated in fig. 3. The animal was made to breathe through a tube, the part nearest to the trachea being at first filled with coarse sand and in another trial a few minutes later with soda lime. The extra dead space in both cases was therefore the same, and the only difference was that in the first instance carbon dioxide accumulation took place simultaneously with the decrease in oxygen percentage. From the fig. it can be seen that the rise in the pressure is the same in both cases.

Ergotamine given in varying doses of 0.1—0.5 mg per kg did not prevent the effect of oxygen and oxygen want respectively on the pulmonary arterial pressure (fig. 4). In most experiments ergotamine seemed even to enhance the oxygen effect somewhat. With regard to the effect of carbon dioxide, however, a reduction was observed after ergotamine, sometimes a slight reversal was found. Similar results were obtained with dihydroergotamine 0.15 mg per kg.

Yohimbine, which has been found by HAMET and VIGNES

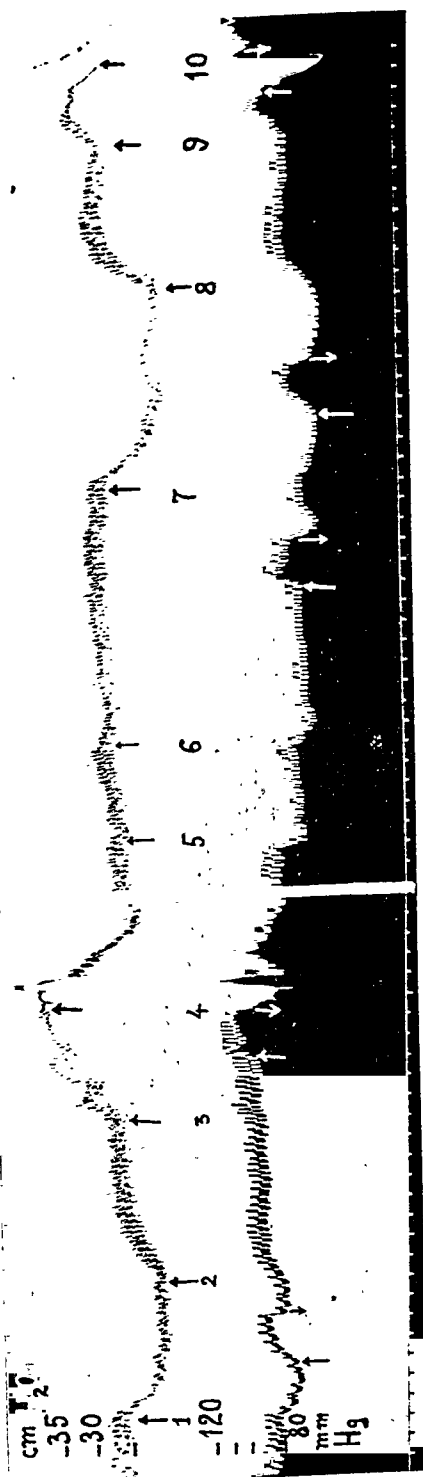


Fig. 5. Cat 2.8 kg. Chloralose. Upper curve pulmonary arterial pressure, lower curve systemic blood pressure. 1. O_2 (from air). 2. Air. 3. 7 p. c. O_2 in N_2 . 4. O_2 . 5. Injection of 3 mg atropine sulf. 6. Inj. of 2 mg atropine sulf. 7. O_2 . 8. Air. 9. 7 p. c. O_2 in N_2 . 10. O_2 . Arrows at lower curve indicate occlusion and opening of common carotid arteries. Artificial respiration. Time 30 sec.

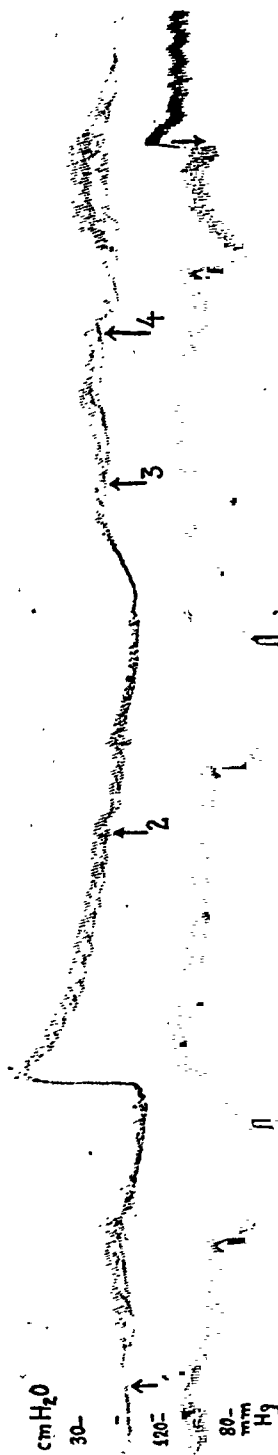


Fig. 6. Cat 2.8 kg. Chloralose. Upper curve pulmonary arterial pressure, lower curve systemic blood pressure. 1. Air. 2. O_2 . 3. Air. 4. 7 p. c. CO_2 in O_2 . Arrows at lower curve indicate increasing ventilation and restoring to normal. Time 30 sec.

(1926) to reverse in dogs the adrenaline effect on the systemic blood pressure, did not in a dosage of 0.7—2 mg per kg in any way affect the changes brought about by pure oxygen, 7 per cent oxygen in nitrogen and 7 or 14 per cent carbon dioxide in oxygen.

Nor did *atropine* in a dose of 1—6 mg per kg influence the response of the pulmonary blood pressure to oxygen, oxygen want or carbon dioxide (fig. 5).

2. Effect of Occlusion of the Carotids on the Pulmonary Arterial Blood Pressure.

In their paper EULER and LILJESTRAND during occlusion of the carotids hardly saw any influence on the pulmonary arterial blood pressure. Since this point has special interest it has been investigated more closely. The effect of the occlusion of both carotids was tested with the animal breathing alternately air, pure oxygen, 7 per cent oxygen in nitrogen and 14 per cent carbon dioxide in oxygen. Usually no effect was found (fig. 4), but in some instances an effect on the pulmonary arterial blood pressure was recorded (fig. 5), especially during oxygen want, a certain decrease then usually appearing, it seems possible, however, that this is secondary, due to a diminution of the blood volume expelled by the right ventricle.

In confirmation of the results of WRIGHT (1930) and of EULER and SCHMITERLÖW (1944) after ergotamine only small pressor responses in the systemic pressure were observed. The same was found for dihydroergotamine in agreement with the observations of EULER and HESSER (1947).

Neither *atropine* nor *yohimbine* had any effect on the pressor response to carotid occlusion.

It may be mentioned in this connection that the increase in the pulmonary arterial blood pressure that follows after occlusion of the artery to one of the lungs, was not influenced by ergotamine. The occlusion before ergotaminization led to a pressure rise from 25 to 33 cm (32 per cent). After ergotaminization, the corresponding values were 30 and 38 cm (increase 27 per cent). Neither did vagotomy, in any way, influence the result of this occlusion. After vagotomy the occlusion of the same artery caused a rise from 30 to 38 cm.

3. Effect of Increased Ventilation.

It is well known that increased ventilation decreases the systemic blood pressure to a considerable degree, partly through mechanical influence, and partly by washing out some of the carbon dioxide. In the experiments described in this paper decreases up to 75 per cent have been recorded. The influence of increased artificial ventilation on the pulmonary arterial blood pressure in comparison with the effect on the systemic pressure is illustrated in fig. 6.

The immediate effect of the increased ventilation was a marked drop in the systemic pressure, followed by a continuous slow reduction. The pulmonary arterial blood pressure was very little influenced at the beginning, then it showed a small reduction. As soon as normal ventilation was restored, the systemic pressure rose to the previous level, at first quickly and then at a fairly slow rate. The pulmonary arterial blood pressure, on the other hand, after about one minute showed a rapid rise and attained a value above the level before ventilation was increased. If the artificial ventilation was increased in exactly the same way as before but oxygen was substituted for air, the curve for the systemic pressure had the same appearance as before. The pulmonary arterial blood pressure, however, was only very little influenced. Especially the rise, after ventilation had been reduced again to normal, was now practically absent. This shows that whereas the slow lowering of the systemic pressure is caused by the washing out of carbon dioxide, this is not the case for the pulmonary arterial blood pressure. But the increased ventilation not only leads to a washing out of carbon dioxide, it also causes a higher alveolar oxygen tension and thereby induces a drop in the pulmonary arterial blood pressure. With the restoring of normal ventilation the alveolar oxygen tension is lowered again, and the pressure in the pulmonary artery rises. The last experiment in fig. 6 demonstrates that the systemic pressure now only displayed the primary lowering but not the secondary one, when washing out of carbon dioxide was prevented by giving 7 per cent carbon dioxide in oxygen. The pulmonary arterial blood pressure was not affected at all except that bigger waves typical of the increased respiration appeared. From the experiments the conclusion seems to be warranted that carbon dioxide has a much smaller effect on the tone of the pulmonary arterioles than on the corresponding vessels in systemic circulation.

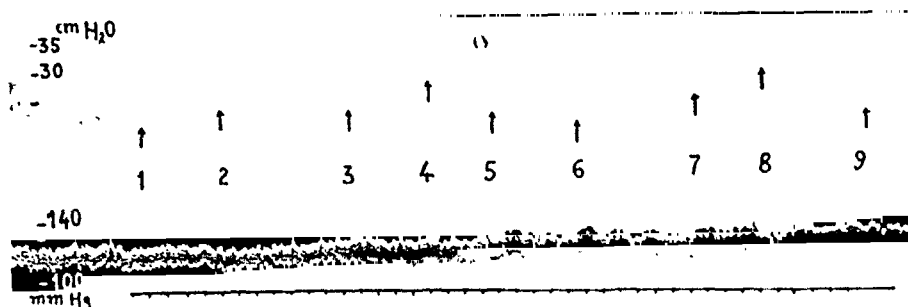


Fig. 7. Cat 3.4 kg. Chloralose. Upper curve pulmonary arterial pressure, lower curve systemic blood pressure. 1. Increase by 1 cm of the expiratory resistance. 2. Further increase by 0.5 cm of the expiratory resistance. 3. 1.5 cm increase of the inspiratory resistance. 4. Inspiratory resistance restored to normal. 5. Expiratory resistance restored to normal. 6. 1.5 cm increase of the inspiratory resistance. 7. 1.5 cm increase of the expiratory resistance. 8. Expiratory resistance restored to normal. 9. Inspiratory resistance restored to normal. Spontaneous breathing. Time 30 sec.

4. Effect of Increased Resistance to Respiration.

When the animal is breathing spontaneously through Müller valves, an increase of the resistance on the inspiratory or the expiratory side affects the pulmonary arterial blood pressure; in both cases a rise takes place.

Fig. 7 gives an illustration. At the start the pulmonary arterial blood pressure was 24.5 cm. When the expiratory resistance was elevated 1 cm, the blood pressure rose to 27 cm. A further increase of the expiratory resistance with 0.5 cm brought the blood pressure in the pulmonary artery to 31.3 cm from which it dropped to 27 cm. Now the resistance during inspiration was increased 1.5 cm; the pressure in the pulmonary artery rose to 32.5 cm. Restoring the inspiratory resistance to normal (1—2 mm), the pulmonary arterial pressure dropped from 31.7 to 25.7 cm and restoring the expiratory resistance to normal it fell from 25.7 to 22.5 cm. The elevation during increased expiratory pressure is mainly due to oxygen want as shown in the following experiment. With the animal breathing air the pulmonary arterial pressure was 32.5 cm. An increase of the expiratory resistance with 1.4 cm caused a rise in the pulmonary arterial pressure to 37.2 cm, and after restoration to normal the value was 33.5 cm. Under oxygen breathing the same respiratory resistance only led to an increase in the blood pressure from 32.8 to 34 cm (fig. 8).

It should be emphasized that the rise in the pulmonary arterial

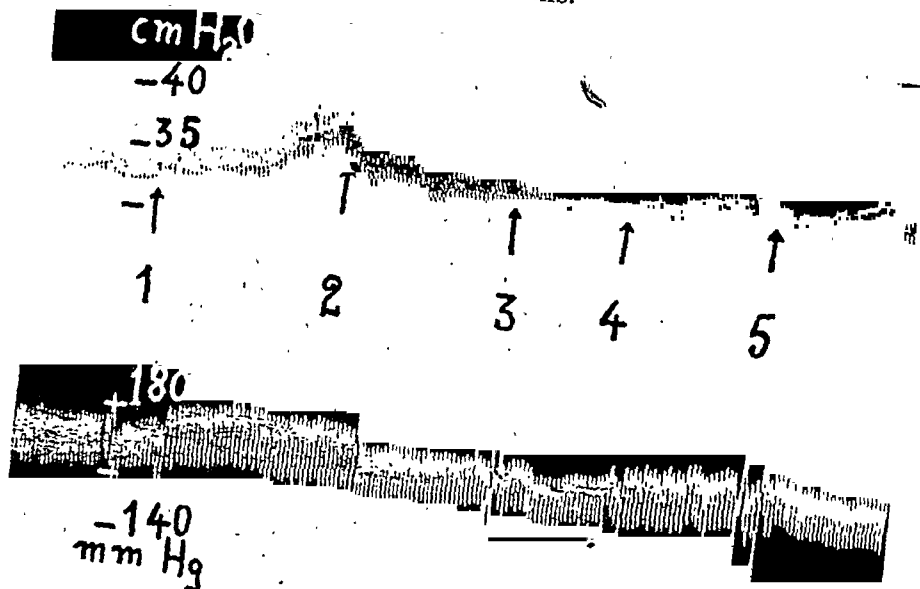


Fig. 8. Cat 3.2 kg. Upper curve pulmonary blood pressure, lower curve systemic blood pressure. 1. Increase of the expiratory resistance by 1.5 cm. 2. Restoration of the expiratory resistance to normal. 3. Oxygen breathing. 4. Increase of the respiratory resistance by 1.5 cm. 5. Restoration of the respiratory resistance to normal. Closed thorax. Spontaneous respiration, Time 30 sec.

pressure is considerably higher than the increase in the resistance on the expiratory or inspiratory side.

An increase of the pressure in the lungs during the whole respiratory cycle was obtained by placing a suitable weight on the gas bag and adjusting it so that the gas was slowly driven through the Müller valves. This corresponds to the positive pressure apparatuses used in human therapy. As seen from fig. 9, the pulmonary arterial pressure was unaffected.

5. Effects of Various Drugs on the Pulmonary Arterial Pressure.

a) *Adrenaline*. A number of experiments were performed with small or moderate amounts (0.005—0.02 mg) of adrenaline. In agreement with EULER and LILJESTRAND a slight or marked temporary rise in the pulmonary arterial pressure was observed, the pressure in the left auricle remaining constant or even being somewhat lowered. The result therefore cannot be due to back pressure, as found by JOHNSON, HAMILTON, KATZ and

WEINSTEIN (1937), as well as by HAMILTON, WOODBURY and VOGT (1939) after large doses. As a matter of fact after the small doses used here the gradient from the pulmonary arterial pressure to the left auricle pressure rose about 30 per cent in several instances. Since doubling of the blood flow is

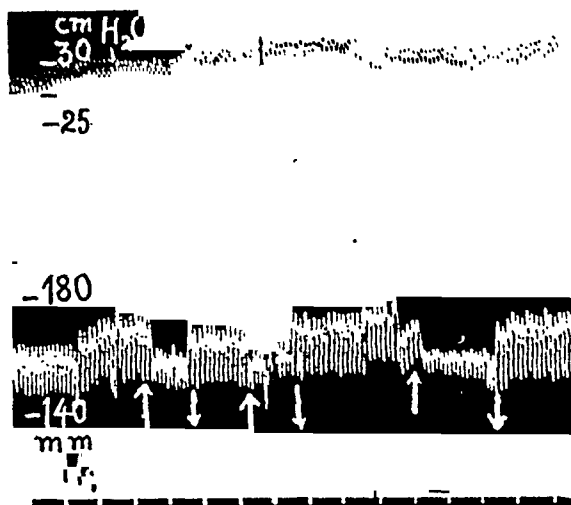


Fig. 9. Cat 3.2 kg. Upper curve pulmonary arterial pressure, lower curve systemic blood pressure. Arrows indicate increase and restoration to the normal of the pressure in the lungs. Closed thorax. Spontaneous breathing. Time 30 sec.

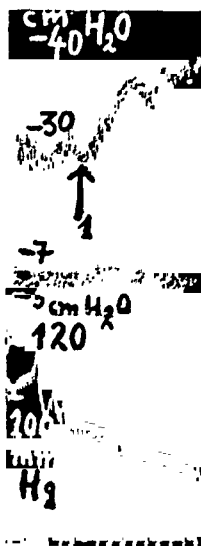
necessary to cause such a rise in the pulmonary arterial pressure (cp. EULER and LILJESTRAND and the experiment mentioned p. 127), it seems hardly probable that the observed rise can be wholly explained by an increased blood flow. A direct influence on the pulmonary vessels seems likely.

After ergotamine or yohimbine a reversal of the effect of adrenaline on the pulmonary arterial pressure was noted.

b) *Ergotamine*. MELLIN (1904) first noticed a marked increase of the pulmonary arterial pressure in rabbits after injection of fluid ergot extract. WIGGERS (1911) recorded the same effect after ergotoxine. MELLIN attributed this increase of the pulmonary arterial pressure to vaso-constriction of the lung vessels. WIGGERS has shown that ergotoxine can also cause an augmentation of the activity of the heart after a primary depressant action. In the experiments recorded here ergotamine tartrate (0.15 mg per kg) regularly increased the pulmonary arterial pressure, sometimes enormously, whether the carotid pressure

was rising or falling. Sometimes the increase was several hundred per cent (fig. 4). In one instance the pulmonary arterial pressure after 0.3 mg ergotamine tartrate per kg rose from 22.5 to 62.5 cm. The elevated pressure persisted for an extended period. It is clearly impossible that this great affect can be due to an increase in the blood flow alone. Neither is the effect to be attributed to back pressure from the left auricle, since the pressure in the auricle

Fig. 10. Cat 29. kg. Chloralose. Upper curve pulmonary arterial pressure, middle curve left auricle pressure, lower curve systemic blood pressure. 1. Injection of 0.3 mg ergotamine tartrate. Closed thorax. Spontaneous breathing. Time 10 sec.



was not at all or very little increased. It seems necessary to assume a constricting action of the arterioles in the lungs.

On the other hand dihydroergotamine tartrate had little or no effect on the pulmonary arterial pressure although the effect on the systemic blood pressure was considerable. Doses of 0.1—0.15 mg per kg led to a great fall of the systemic pressure with no or only a small effect on the pulmonary arterial pressure.

c) *Yohimbine*. In all cases the systemic as well as the pulmonary arterial pressure fell after the administration of 0.7—2.0 mg of yohimbine hydrochloride per kg. This is probably due to vasodilation. But when doses of 0.2 mg per kg were repeated both pressures showed very little effect, but sometimes both pressures displayed a temporary increase.

d) *Atropine*. After atropine the systemic blood pressure was decreased and at the same time the pulmonary arterial pressure showed a small increase.

e) *Pilocarpine*. After administration of pilocarpine a decrease of the pulmonary arterial pressure was noted. The effect is similar to the one observed by EULER and LILJESTRAND after acetylcholine.

Discussion.

The results obtained with certain drugs that are known to act on the autonomic innervation of different organs, seem to indicate that the tone of the lung vessels might, to some extent, be controlled through these systems. No evidence has been obtained, however, that such is the case during physiological conditions. Clamping of the carotids which profoundly influences the splanchnic outflow, only exercises a very small effect on the pulmonary arterial pressure which may well be secondary, and the rise in pressure after occlusion of the branch of the pulmonary artery to one lung was neither affected by vagotomy nor by ergotaminization.

An increase in the alveolar oxygen tension leads to a drop and a decrease to a rise in the pulmonary arterial pressure. These effects are not related to simultaneous reactions from the systemic pressure which may or may not be in the same direction. When the pulmonary arterial pressure rises during moderate oxygen want, no increase in the pressure in the left auricle was found. Therefore the rise of pressure in the pulmonary artery is not due to back pressure from the left heart. Nor is it caused by an increased blood flow, for even a doubling of the flow leads to a very moderate increase of the pressure (20 to 30 per cent), whereas moderate oxygen want will often increase the pulmonary blood pressure a hundred per cent or more. This rise must be the result of a constriction of the pulmonary vessels themselves.

The effects of variations in the oxygen tension on the pulmonary blood pressure seem to be independent of the innervation of the lung vessels. Thus EULER and LILJESTRAND found that neither vagotomy nor extirpation of the stellate ganglia had any influence on the effects of oxygen tension on the pulmonary arterial pressure. The experiments communicated in this paper with ergotamine, yohimbine and atropine point clearly in the same direction. It must be concluded that the lung vessels are in some way able to react themselves for the variations in the oxygen tension.

With regard to carbon dioxide the situation is less clear. An

increased carbon dioxide tension may cause a constriction of the lung vessels, but the effect is only small. After ergotaminization it may be abolished or even reversed. This would seem to imply that the effect of the carbon dioxide is at least in part exercised through the mediation of the autonomic nerves.

It has been pointed out earlier (EULER and LILJESTRAND) that the influence of oxygen tension on the pulmonary vessels would seem to constitute a regulating mechanism for the adequate distribution of the blood flow through the different parts of the lungs. This view is strengthened by the observations made in this paper. There seems to be no doubt that the effect is of a local nature.

Summary.

It has been confirmed that the pulmonary arterial blood pressure is increased by inhalation of gas mixtures poor in oxygen and decreased if pure oxygen is breathed. The pressure in the left auricle need not be effected at all, so that the tonus of the arterioles in the lungs must become influenced by the oxygen tension. That this is due to a local effect, as concluded from earlier experiments (EULER and LILJESTRAND), is strengthened by the fact that it is not abolished by ergotamine, dihydroergotamine, atropine or yohimbine.

Increased resistance during inspiration or expiration leads to a rise in the pulmonary arterial blood pressure. Oxygen inhalation greatly reduces this effect of increased expiratory resistance. Increased pressure in the lungs during the whole respiratory cycle left the pulmonary arterial pressure unaffected.

Carbon dioxide accumulation may also cause a rise of the pulmonary arterial pressure, but the effect is small and can be abolished by ergotamine. Reasons are given for the conclusion that the carbon dioxide tension has a much smaller effect on the tone of the pulmonary arterioles than on the corresponding systemic vessels.

Occlusion of the carotids had only a slight effect on the pulmonary arterial pressure which might be secondary.

After small doses of adrenaline the pressure gradient between the pulmonary artery and the left auricle rose about 30 per cent. After ergotamine a very great increase of the pulmonary arterial pressure with no or only slight effect on the pressure in the left auricle was obtained. It seems necessary to assume that these drugs cause a constriction of the arterioles of the lungs.

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The Thyroid-Ovarian Correlation in the Rabbit.

By

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The glands that make up the endocrine system are intricately interrelated. A unique position is occupied by the thyroid. The clinical and experimental problems presented by this gland were taken up for study at an early stage, and numerous investigations have been published on the relationship between the thyroid and the other endocrine glands. It is generally agreed that, in the female, functional disorders of the thyroid are often accompanied by irregularities in the sexual cycle. Opinions differ, however, as to the nature of these irregularities and their connection with the thyroid function.

Experimental investigations have been conducted with hypo- and hyperthyroidism in the ordinary laboratory animals in order to elucidate the effect of the thyroid on the ovarian function.

HOFMEISTER (1894) seems to be the first to have studied the effect of thyroidectomy with special reference to the ovaries. In experiments on rabbits, he frequently observed degeneration in the ovaries, sometimes manifested as "follikuläre Hypertrophie" and sometimes as "generalisierte Follikelschrumpfung". BIEDL found pronounced hypoplasia of the ovaries in thyroidectomized dogs and cats.

In ten experiments on dogs and cats, FRIEDMANN (1933) showed that insemination, gestation and lactation were possible in the complete absence of the thyroid. Other workers have described various complications of pregnancy in thyroidectomized animals.

TATUM (1913) observed degenerative changes in all the parenchymatous organs, particularly the endocrine organs, in thyroidectomized rabbits. He noted absence of development of the sex elements with degeneration of the germinal epithelium in the ovaries.

SEITZ and LEIDENIUS (1925) found that the animals could become pregnant if small remnants of the thyroid were left in place.

KUNDE, CARLSON and PROUD (1929) reported degeneration of the graffian follicles following thyroidectomy.

KNAUS (1924) found that guinea-pigs could be thyroidectomized with no effect on gestation.

LEE (1925) thyroidectomized rats and observed a prolongation of the cycle by about one day for five months. There was no effect on the age of puberty.

EVANS and LONG (1921) were unable to find any effect on the estrous stage or the onset of puberty in rats. Nor could they observe changes in the estrous stage following the administrations of thyroid preparations.

GRUMBRECHT (1920), experimenting with rats, reported that the changes in the cycle and in the ovaries may be the same in hyperfunction as in hypofunction. He observed signs of increased maturity of the ovum following thyroid treatment. He considered that the sensitivity of the ovary to the gonadotropic hormone is changed by thyroid medication.

LEOPOLD and LEISSER (1923) studied the effect of hyperthyreosis in rabbits and found pronounced follicular degeneration after 13, 17 and 22 days of thyroid medication.

ENGELHART (1935) investigated the function of the corpus luteum in rabbits after the administration of a thyroid preparation. He gave the animals thyroid powder from the second and the third day after sterile coitus. Examination of the ovaries 11, 12, 13, 14, 15 and 16 days later revealed that regeneration of the corpus luteum began on the eight to the tenth day and was very pronounced by the fourteenth day. On the thirteenth day the reaction of the uterine muscle to pituitrin had again become positive. ENGELHART stressed the contrast with normal cases, in which complete degeneration of the endometrium cannot be observed until the seventeenth day.

In later experiments in 1942, ENGELHART found that hyperthyroidism in rabbits led to premature termination of pregnancy

and failure of the function of the corpus luteum. He considered he could show a certain antagonism between the thyroid gland and the corpus luteum hormone. If the experiment was made with a sterile uterine horn, the mucosa exhibited premature degeneration. He made the same observation in experiments with pseudopregnant rabbits. Here the muscular reaction was positive and the endometrium was at rest on the fifteenth day and degeneration of the endometrium was discernible by the thirteenth day.

No change in the cycle in the guinea pig results from thyroid medication. If the animal is pregnant, however, it reacts to thyroid treatment by aborting. Some workers consider that the death of the fetus is primary, while others believe that the abortion itself comes first. In young guinea-pigs, prolonged treatment results first in hyperemia, later in proliferation in the ovaries, and finally in cessation of follicular growth.

Numerous other workers (SHERWOOD 1940, LAQUEUR and EMGE 1941, KNAUS 1923, ALEXIN 1939, ANDERSSON and KENNEDY 1923, PINEUS and WERTHESEN 1933, DESSAU 1937, KAKUSHKIN and SOLOVEY 1937, *et al.*) have studied changes in the thyroid in castrated animals and following medication with ovarian hormones. Most of them consider that castration is followed by atrophy and reduced activity of the thyroid. Treatment with ovarian hormones is said to lead to both hyperplasia and increased activity. The details of these experiments are of little interest in this connection.

The experiments have thus given contradictory results and have yielded no conclusive evidence of the effect of the thyroid on the ovarian function.

We have conducted a series of experiments on rabbits to discover whether any change in the action of the ovarian hormones can be observed both following removal of the thyroid and the administration of thyroid by injection.

Methods and Results.

The animals used were adult female rabbits which had not previously had young and which had been isolated prior to the experiments. The thyroid preparation was thyranon, manufactured by the Pharmacia Company in Stockholm. The experimental conditions appear in Tables I and II.

Table I.

Exp. No.	Sterile coitus. Days after thyroidectomy	Ovary and endometrium			
		7 days after sterile coitus		14 days after sterile coitus	
		Ovulation	No ovulation (normal)	Degeneration of corp. lut. and endometrium	Normal
1	0	x		+	
2	0		x		
3	2	x		+	x
4	0	x		+++	
5	2	x		died	
6	0	x		+++	
7	10	x		died	
8	0	x		(+)	
9	0		x		x
10	2		x		x
11	2	x		+++	
12	14		x		x
13	2		x		x
14	10	x		(+)	
15	8	x		(+)	
16	10	x		+++	
17	14	x		+++	
18	5	x		+++	
19	10		x	++	
Controls					x
20	0	x		++	
21	10	x		+	

Abbreviations: The sign x shows the reaction of the animal
 (+) insignificant
 + beginning mild
 ++ distinct beginning
 +++ fairly pronounced

Table I covers 19 experiments with thyroidectomized animals. In Nos. 1, 2, 4, 6, 8, and 9 the animals were thyroidectomized within two hours of sterile coitus. In Nos. 3, 5, 10, 11, and 13, two days elapsed between thyroidectomy and sterile coitus, in No. 18 five days, in No. 15 eight days, in Nos. 7, 14, 16, and 19 ten and in Nos. 12 and 17 fourteen days. No ovulation occurred in six of the experiments (nos. 2, 9, 10, 12, 13, and 19). In these cases the ovaries were normal with large follicles and normal estrin action on the uterus seven days after sterile coitus and 7 (two cases), 9, 17, and 21 days after thyroidectomy. In the remaining ovary and uterine horn the same picture could be observed for a further seven days. Ovulation occurred in the remaining 13 cases (corpus luteum formation, secretory phase). One ovary

and part of the uterus were removed seven days after sterile coitus and the other ovary and the uterine horn 14 days thereafter in these cases.

After seven days all the cases exhibited normal corpora lutea in the ovaries and normal progesterone action in the endometrium.

Fourteen days after sterile coitus, Nos. 4, 6, 11, 16, and 17 showed fairly pronounced degeneration of the corpus luteum and beginning degeneration of the endometrium. The remaining 7 cases (1, 3, 8, 14, 15, and 18) exhibited beginning or insignificant degeneration of corpus luteum and endometrium. The ovaries and the endometrium presented approximately the same picture as those of the control animals.

In two cases, Nos. 20 and 21, control experiments consisted only of exposure of the thyroid. In No. 20 sterile coitus occurred on the same day as the operation and in No. 21 ten days after it. In both cases the ovaries showed normally developed corpora lutea and endometrium seven days after sterile coitus. No. 20 showed beginning degeneration of the corpora lutea and endometrial degeneration fourteen days after sterile coitus, while No. 21 only exhibited signs of beginning degeneration of both corpora lutea and endometrium.

It is scarcely possible to draw any conclusions from these experiments as to the effect of thyroidectomy on the action of the ovarian hormones in the uterus. Nos. 4, 6, 11, 16, and 17, it is true, showed somewhat more pronounced degeneration of the corpora lutea and the endometrium than is normal. Certain variations in biological experiments must be taken into account, however, and experience has taught us to expect variations under normal conditions also.

Table II covers 8 experiments (Nos. 22—29) in which the animals were first thyroidectomized and then treated with thyranon in injections of 2 ml every other day for the rest of the experimental period. Sterile coitus occurred two days (Nos. 22, 26, and 28), seven days (No. 24), ten days (Nos. 23 and 25) and fourteen days (Nos. 27 and 29) after thyroidectomy. Two of these animals did not ovulate. The others showed normal corpora lutea and normally developed endometrium seven days after sterile coitus and beginning degeneration of the corpora lutea and the endometrium fourteen days later.

In the four experiments (Nos. 30—33) the animals were given thyranon every other day [1 ml dose in two cases (No. 30 and 31)

Table II.

During the whole experimental time the rabbits were treated with Thyranon. Doses see page 005—006.

Abbreviations see table I.

Exp. No.	Sterile coitus. Days after thyroidectomy	Ovary and endometrium			
		7 days after sterile coitus		14 days after sterile coitus	
		Ovulation	No ovulation (normal)	Degeneration of corp. lut. and endometrium	Normal
22	2		x		x
23	10	x			(+)
24	7	x			(+)
25	10	x			(+)
26	2		x		x
27	14	x			(+)
28	2	x			(+)
29	14		x		died
	Sterile coitus. No thyroidectomy				
30	"	x		++	
31	"	x		+	
32	"	x		++	
33	"	x		++	

and 2 ml dose in two] after sterile coitus. All four ovulated. Seven days after sterile coitus they all exhibited normally developed corpora lutea and endometrium, and fourteen days afterwards they showed degeneration of the corpora lutea and beginning endometrial degeneration.

The experiments 34—36 cover three cases of thyroidectomized mature females. Examination of the ovaries and the uterus 14 and 30 days (No. 34), 30 and 60 days (No. 35) and 60 and 86 days (No. 36) after thyroidectomy revealed no changes (normal ovaries) with normal-sized follicles. Estrin action on endometrium.

In experiments 37, 38, 39 and 40 the animals were given 1 mg of progesterone daily for four days beginning 14 days after thyroidectomy in two cases and 30 days afterwards in two cases. The endometrium in all four showed normal progesterone action (strongly pronounced, complete secretory phase) and resembled in detail the endometrium in case 41, in which the thyroid was only exposed.

Discussion.

As appears from the tables ovulation did not occur in 6 of 19 thyroidectomized animals. Eight cases were given thyranon treatment following thyroidectomy, and three of them failed to ovulate. Of six animals not submitted to thyroidectomy, all of them ovulated after sterile coitus. It is noteworthy that so many of the thyroidectomized animals, or approximately one-third, did not ovulate after sterile coitus. We have no explanation to offer for this. The absence of the thyroid can not have influenced ovulation directly, because, as appears from the tables, several animals ovulated in connection with sterile coitus from one to several days after thyroidectomy.

Summary.

An experimental study was made to determine the significance of the thyroid to the action of the ovarian hormones on the endometrium. The experiments were conducted on pseudopregnant rabbits, both following removal of the thyroid and after the administration of a thyroid preparation. No changes could be demonstrated in the action of the ovarian hormones on the endometrium after thyroidectomy and thyroid medication.

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On the Toxic Effect of Tannic Acid with Reference to the Treatment of Burns.

By

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The coagulating treatment of burns with tannic acid, to judge from the Anglo-American literature of the subject, seems very quickly to have lost the leading position it has enjoyed for a couple of decades. One must reckon with two different causes of this very striking revaluation of a form of treatment that has been acknowledged over the whole world. It has been shown clinically and experimentally that *tanning of a wound* always delays the physiological healing. To this may be added the experiences from the medical field work of the second World War, where it proved that the tanning of unclean burns and burns of the third degree gave such bad results that the method was forbidden in certain armies. Furthermore, *tannic acid has been described as a toxic substance for the liver*, whereas it was previously regarded as directly life-saving through its effect on the burned surface.

Cases of necrosis of the liver in connection with burns have been known for about ten years, but it was WILSON, Mc GREGOR and STEWART's publication in 1938 concerning injuries to the liver in the toxic stage of burns that gave rise to the lively discussion of these questions that has since been carried on. From several quarters we have had descriptions of severe icterus after burns and necrosis of the liver in post-mortem cases. These injuries to the liver were especially observed in those cases that had died 2—8 days after a burn, and they were patho-anatomically de-

scribed as fatty degeneration or complete necrosis of the epithelial cells around the veins in the lobuli of the liver. Concerning the mode of origin of these injuries to the liver, opinion was divided. WILSON and his colleagues saw therein a definite proof of the presence of a burn toxin, but others regarded them as among the phenomena caused by shock from burns, with its anoxemia and pathological vascular permeability.

The burn cases that on post-mortem showed these central liver necroses had all been locally treated with tannic acid, *and it was clinical material that gave rise to the suspicion that tannic acid might be a toxic substance that after absorption had a special affinity for the liver.* Experiments were undertaken by WELLS (1940), who was able to show central necroses of the liver in rabbits after intraperitoneal or subcutaneous injections of tannic acid. This communication of course aroused very great interest, and investigations were started in several quarters in America and England. In 1942, WELLS, HUMPHREY and COLL published four cases of tannic acid-treated burns with necroses of the liver. Similar necroses could be produced in rats by subcutaneous injections of tannic acid. CAMERON and co-workers (1943) were able to show in experiments on 250 animals of different kinds that intravenous injections of tannic acid were fatal, and that cardiac dilatation and injuries to the liver and kidneys arose as a result. If the injections were given intramuscularly and subcutaneously a considerably greater amount of tannic acid was required. In this connection it was also found that a pronounced edema arose at the site of the injection, and that a hemo-concentration and a fall in serum protein followed. After the tannic acid treatment of a burn it was possible to demonstrate tannin in the blood (ferri-chloride reaction), and in a number of the animals injuries to the liver were shown (not after subcutaneous injections). BARNES, ROSSITER and CLARK (1943) arrived at similar results. The fatal dose for guinea-pigs was found to be 40 mg tannin/kg on intravenous injection, but the dose was considerably higher on intramuscular injection. After the application of tannic acid to the burned surface it was possible to demonstrate tannic acid in the internal organs, and slight injury to the liver was observed. In 1943 BAKER and HANDLER showed necroses of the liver in rats after subcutaneous injections of tannic acid. The absorption of tannic acid from burns could not be investigated, as burned surfaces resembling those in man could not be

produced on animals. Tannic acid in other wounds could bring about injuries to the liver. In 1943 HARTMAN and ROMENCE obtained slight injuries to the liver in burned animals, but severe injuries if the burned surface had been treated with tannic acid, lapis or iron chloride. In 1941 HARTMAN, together with BUIS, found severe liver-injuries in 4 out of 5 cases of fatal burns. ERB, MORGAN and FARMER (1943) found in a post-mortem material of 61 cases that had died of burns that 60 % of the 41 cases that had been treated with tannic acid had liver-injuries of different degrees, while those cases that had been treated without tannic acid did not show any necroses of the liver.

The experimental investigations mentioned here seem to have been accepted as completely valid, as they were considered to confirm the suspicions of the clinicians that the tannic acid was the cause of the necroses of the liver in connection with extensive burns. It is, however, possible to raise a number of objections to this conclusion. The majority of the hitherto published cases of liver-necrosis after burns have, certainly, arisen in cases that had been locally treated with tannic acid; but during these years the tanning of burns was the leading treatment, so that it is only from the most recent years that a large material for comparison may be expected. In 1944 KOCH reported that since the tannic acid treatment had been abandoned in 1938 no cases of liver-necrosis had been observed, and that the mortality had been considerably reduced. In other quarters, however, injuries to the liver have been observed after indifferent treatment of the burned surface, *e. g.* at the Medical Research Council's Burns Unit in Glasgow.

Of recent years great attention has been paid to the liver changes in connection with different states of shock. It has been shown that typical central liver-necroses may arise in connection with the most various states of shock. Central necroses of the liver have for example been produced by RUBARTH (1945) in connection with anaphylactic shock in dogs. In anoxemia also the hepatic capillaries become pathologically permeable, the intercellular pressure is increased and the parenchyma cells are damaged chiefly in the central parts of the acini. (RÖSSLE, EPPINGER 1935, MOON 1942 and others.).

The typical shock from burn is characterized precisely by pathological capillary permeability, which renders possible injuries to the parenchyma of the liver. *It must thus be stated that liver-*

necroses may be conceived to arise in connection with extensive burns independently of the local treatment that has been given.

It must be considered as very remarkable that the necroses of the liver that have arisen in experiments on animals after injections of tannic acid have identically the same appearance as the liver-necroses in different states of shock. It is thus first of all necessary to prove that tannic acid in a certain concentration in the blood acts toxically upon the liver. This seems also to have been established by the previously mentioned experiments on animals where the tannic acid has been administered intravenously. It should, however, be remarked that in none of these series of investigations has the blood concentration been given. — In connection with subcutaneous injections of tannic acid all the writers give considerably higher amounts of tannic acid for the production of liver-necroses. It should here be observed that a strong local reaction with necroses and abscesses always appears in connection with subcutaneous and intramuscular injections, and that veritable states of shock with hemo-concentration and fall of serum protein have been observed. Blood analyses after subcutaneous injections have not, however, been given.

Experimental burns on animals have not the same appearance as burned surfaces on man. Thus, for example, blisters or weeping surfaces cannot be produced on animals, that instead show a dry, necrotic surface resembling a burn of the 3rd degree in man. These burns mean that the circulation is seriously affected, and that as a rule thromboses have arisen in the skin vessels. It can therefore not be expected that substances can be resorbed through such surfaces. Also in connection with the typical burning of the 2nd degree in man the conditions of resorption have not been explained. Since UNDERHILL's (1930) investigations it has long been considered that scarcely any resorption occurs, but this view has now been revised, as it has been shown in experiments with sulfa preparations in local treatment that a very high sulfa-concentration in the blood may arise.

A number of researches now consider themselves able to show that injuries to the liver have appeared after the treatment of experimental burned surfaces in animals with tannic acid. Very little importance can be attached to these investigations, as determinations of the tannic acid-content of the blood have not been carried out and the absorption of the tannic acid has not been explained.

Also in Sweden necroses of the liver have been observed in connection with burns treated with tannin. ROSENQVIST (1945) considered that the proofs against tannic acid as a substance toxic to the liver were so strong that the tannic acid treatment should be abstained from until the question had been gone into more thoroughly. For this reason experiments were undertaken in 1944 according to the following plan:

After the working out of a micro-method for the quantitative determination of the tannic acid-content in the blood plasma, attempts were to be made to produce liver-necrosis in animals by tannic acid injections, and determination of the necessary concentration of tannic acid in the blood was to be performed.

Method.

I. Quantitative determination of tannic acid in blood plasma.

Since tannic acid (in the sequel referred to as HTa) is not a well-defined chemical compound with a known constitution, it has not been possible to indicate the principles according to which a specific determination should be carried out. On the other hand, the phenol groups in HTa could be exploited for unspecific reactions. Thus, for pure solutions quantitative methods have been given that are based upon the reduction of arseno-tungstic acid (KÜNTZEL 1943), of molybdo-tungstic acid (Follin-Ciocalteu's phenol reagent, ROSENBLATT and PELUSO 1941) and of osmium acid (MITCHELL 1924) or based upon colour reactions between phenols and iron salts (MITCHELL 1923). All of these methods are photometric, and therefore in principle usable as micro-methods. After preliminary experiments with different methods we have chosen the arseno-tungstic reaction (KÜNTZEL), which is the most sensitive, and which has proved to be usable for the problem here in question.

Arseno-tungstic reagent: 15 g As_2O_5 , 50 g NaWO_4 , 25 ml conc. HCl and 150 ml distilled water are refluxed for 2 hours. The clear, greenish solution is diluted to 500 ml with distilled water. Should be kept in dark bottle.

The procedure: 1 ml plasma is mixed well with 2 ml reagent. 3 ml 1.65 N soda solution is then added, and after mixing, the sample is allowed to stand in a water-thermostat at 37° for 20 minutes. A blue colour and a white turbidity appear. Centrifuge for 45–60 minutes at the rate of 3,000 rev./min. The clear centrifugate is photometrized in a Pulfrich photometer with filter S. 72 in 0.25 or 0.5 cm cuvette with water as compensatory solution. The extinction is corrected for blank test on plasma taken before the addition of tannic acid, and the HTa concentration is read off from the calibration curve.

The calibration curve is obtained from a standard series, which must be made for each new reagent. In a series of centrifuge tubes 0.100 ml

of different solutions of tannic acid, 0—80 micrograms of HTa and 0.90 ml plasma, serum or water are taken and treated as samples. In table 1 are given the results of such standard series.

Table 1.

Standard series on human plasma + tannic acid.

Amount of HTa in μg per 6 ml	21.2	40.7	81.7
No of analyses	16	20	18
$E_{0.5}^{72}$, mean value corrected for blank	0.254	0.473	0.850
Standard deviation	0.0314	0.0422	0.074
Coefficient of variation	12.4 %	8.9 %	8.7 %

The specific extinction coefficient, k , per mg HTa per ml is generally 120—150. As blue colouring is obtained also with normal plasma, some experiments have been carried out to ascertain the cause of the blank value. It then proved that uric acid gave a specific extinction coefficient $k = 59$, hypoxanthine and xanthine 1.6, guanine 0.6, tryptophane 0.3 and phenol 0.16, while histidine, tyrosin, glycerol, glucose, lactic acid, sodium citrate and heparin had $k < 0.1$. The blank value is generally $E_{0.5}^{72} = 0.200$ —0.450, and an uric acid-content

of 3 mg per 100 ml gives $E_{0.5}^{72} = 0.148$, which corresponds to the extinction in protein-free filtrate. The blank value must therefore be due for the most part to substances that are precipitated on de-proteinization. It is presumably a matter of an unspecific reduction, and the animal experiments have not given evidence of greater variation in this through the addition of tannic acid, so that further experiments on the blank value have not been carried out.

II. Experiments on animals.

For these experiments we have used exclusively rabbits weighing 1.7—3.1 kg. For the taking of blood samples a technique resembling that described by SJÖWALL (1937) has been employed. For the experiments with burns the following technique was used: the skin on one side of the rabbit was epilated with strontium sulphide. After about one hour, when the hyperemia had for the most part disappeared, the animal was anesthetized with a small dose of isopropyl-bromallyl-N-methylmalonylcarbamide-sodium (Narcotal "Astra") intravenously, and the anesthesia was maintained with trichlorethylene on the mask. After this, a round metal box with a diameter of 5 cm, in which water at 85° was

circulated, was applied to the naked skin, being kept for 60 seconds on each spot, until the desired burning had been obtained. The area of the burned surface in relation to the body surface was measured after death from the skins of the flayed animals.

In experiments with prolonged intravenous infusion of tannic acid solution the anesthesia was commenced with urethane intravenously. After this, the infusion cannula was inserted into v. jugularis. The anesthesia was maintained with urethane, 2 % of which was contained in the infusion fluid. This also contained 0.5 ml 5 % heparin per 50 ml in order to prevent coagulation. Fresh tannic acid solutions in 0.9 % Na Cl were prepared daily in all the animal experiments and standard series.

Results.

I. The absorption and excretion of the tannic acid.

As a part of the task we had set ourselves was to determine the mean concentration of tannic acid in the blood required for the production of necrosis of the liver, it was necessary first to try out a plan of dosing. As the subcutaneous injection of tannic acid was considered best to correspond to the conditions obtaining in a burn treated with tannic acid, the tannic acid concentration was first determined in the blood at different times after a single subcutaneous injection of different amounts of tannic acid. The values obtained are given in fig. 1.

From this it emerges that the absorption from a 10 % tannic acid solution is delayed as compared with that from a 5 and 2.5 % solution. This is probably due to the fact that a more extensive and pronounced coagulation of the tissue around the site of injection arises. A more careful analysis of the blood values with reference to the rate of absorption and elimination (cf. TEORELL 1937) was not carried out, as it did not fall within the scope of the present work.

II. Experiments on liver-necrosis.

As the absorption experiments had shown that the greatest part of the tannic acid entering the blood excreted within 12 hours, in the experiments aiming at the production of necrosis of the liver 5 % tannic acid was injected, as a rule every twelve

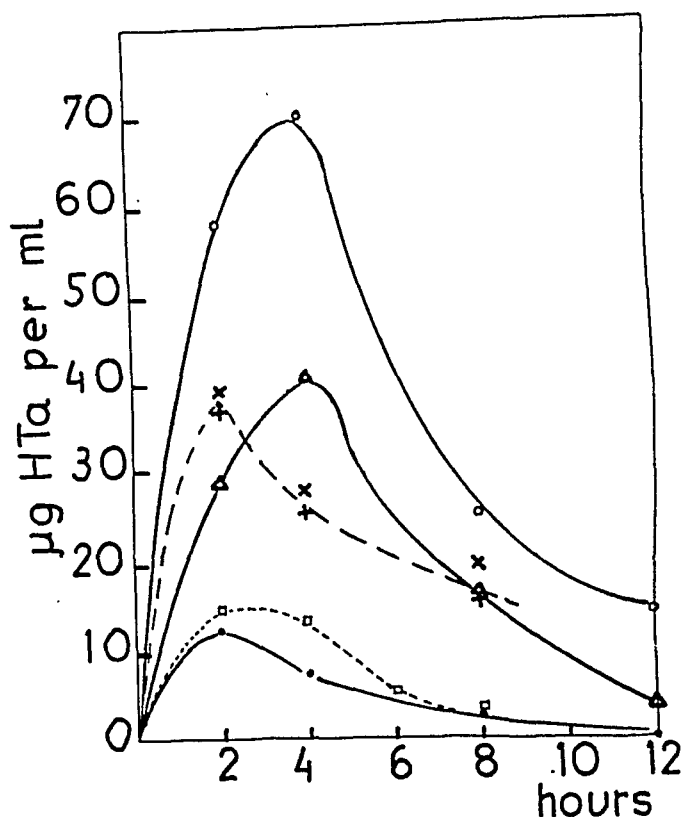


Figure 1.

Mean concentration of tannic acid in micrograms per ml plasma at different times after subcutaneous injection of single doses of tannin.

Dose per kg of body-weight: 125 mg as a 5 % solution (dots); 250 mg as a 2.5 % solution (+); 250 mg as a 5 % solution (x); 250 mg as a 10 % solution (squares); 500 mg as a 5 % solution (circles); 500 mg as a 10 % solution (triangles). No. of animals in each series 2 to 7.

hours, until the animals died. Only 3 of 24 rabbits were unaffected at the conclusion of the experiment, and these were killed. In order to control the absorption of tannic acid, samples were taken 2 hours after subcutaneous injection, and also, where this was possible, at the conclusion of the experiment. The analyses show at first a decreasing resorption, more pronounced the greater the amount of injected tannin is, but at the conclusion of the experiment an apparent rise of the tannic acid values, which, however, as it has the same general trend as the non-protein nitrogen values, is probably due to increased uric acid-content and not to increased absorption of tannin. This is also in accordance with the observation of REICHENAU that tannic acid stimulates the excretion of uric acid in the urine. In addition, determinations of

Table

Subcutaneous injections of 5 % tannic acid.

Animal number	54	55	¹ 56	57	¹ 59
Number of injections	8	7	14	3	11
No. of days	4	4	8	2	7
Time from last inject. to death in hours	<12	6	24	12	48
Conc. of HTa in $\mu\text{g/ml}$ plasma at end of exper.	—	20	20	—	(58)
Total quantity of HTa injected in g/kg bodyweight	1.65	1.45	1.53	0.33	2.25
Liver: Central acinar necrosis s. necrobiosis	0	+	0	0	0
Kidneys: Degenerative changes in tubular epithelium	+	0	0	0	0

protein, non-protein nitrogen and bilirubin in plasma as well as of the hemoglobin-content in the blood were carried out. Of these, the protein-content in plasma, after an initial sinking during the first twenty-four hours, showed a fairly constant level, and the content of bilirubin was always less than 1 mg/100 ml. The non-protein nitrogen, on the other hand, rose regularly after some days (the highest value was 75 mg/100 ml). During the first few days the hemoglobin value was unchanged or moderately reduced; but towards the end of the experiment it sank markedly (to half the original value or less), as did also the blood corpuscle volume.

The experiments with subcutaneous injection of tannin without other treatment are reproduced in tables 2 and 3. The tannic acid values in plasma for a series of experiments are given in fig. 2.

The examinations of the organs showed the following:

(21 of the animals died spontaneously, 3 were killed.) Parts of the liver, the kidney and the myocardium of all the animals were taken and subjected to fixation in a 10 per cent formaldehyde solution. These tissues were stained by means of the ordinary histological technique with Htx-eosin, Htx-v. Giesson, Weigert's fibrin-dye and fat dye with Sudan. The examinations of these cuts were carried out without any knowledge of the quantity of tannic acid that was given to the individual animals.

Changes were observed in the liver as degenerative destructions or necroses of the parenchyme cells within the central parts of the acini. These changes conform with those manifested by persons who have died of burns. Tables 2—5, the extent of the changes is denoted by + — + + +, where + means slight changes, + + + extensive necroses.

¹ killed.

2.
Dose 200—250 mg HTa/kg body-weight.

60 8 4	61 11 7	62 8 4	84 9 5	85 10 6	86 10 6	87 9 5	88 10 6
<12	<36	<12	<12	<12	12	<12	4
31	—	(45)	—	—	—	—	(60)
1.25	2.25	1.65	2.25	2.50	2.50	2.25	2.50
(+)	0	(+)	++	+++	+++	+++	+
0	0	0	+	0	+	+	0

In some cases, *e. g.* Nos. 79, 81 and 83 also fibrinthrombs were seen in the veins. The part played by these thrombs with regard to the genesis of the parenchyme degenerations can not be determined, since they were not found in other animals with about the same degree of parenchyma injury, for instance, Nos 82 and 85.

Finally, coccidiosis was ascertained in a few cases, although not to an extent affecting an estimation of the investigation results. — A few of the animals had slight degenerative changes in the epithelium of the kidneys. No changes at all were found in the myocardium of all these animals.

In order to find out whether the combination of a burn with tannin injections could lead to necrosis of the liver, a series of experiments were carried out with subcutaneous HTa injections, after the animals had been given a 3rd degree burn covering from 5.5 to 14.2 % of the body surface. The results are given in table 4. By way of control, two rabbits (nos. 69 and 70) were burned with-

Table 3.

Subcutaneous injections of 5 % tannic acid. Dose 500 mg tannin per kg of body-weight.

Animal number	79	80	81	82	83
Number of injections	8	8	8	8	7
No. of days	4	4	4	4	4
Time from last inject. to death in hours ..	<4	25	<10	25	<11
Conc. of HTa in μ g/ml plasma at end of exp.	71	43	63	41	—
Total quantity of HTa injected in g/kg body-weight	4.0	4.0	4.0	4.0	3.5
Liver: Central, acinar necrosis s. necrobiosis	++	++	++	+	+++
Kidneys: Degenerative changes in tubular epithelium	+	+	0	0	+

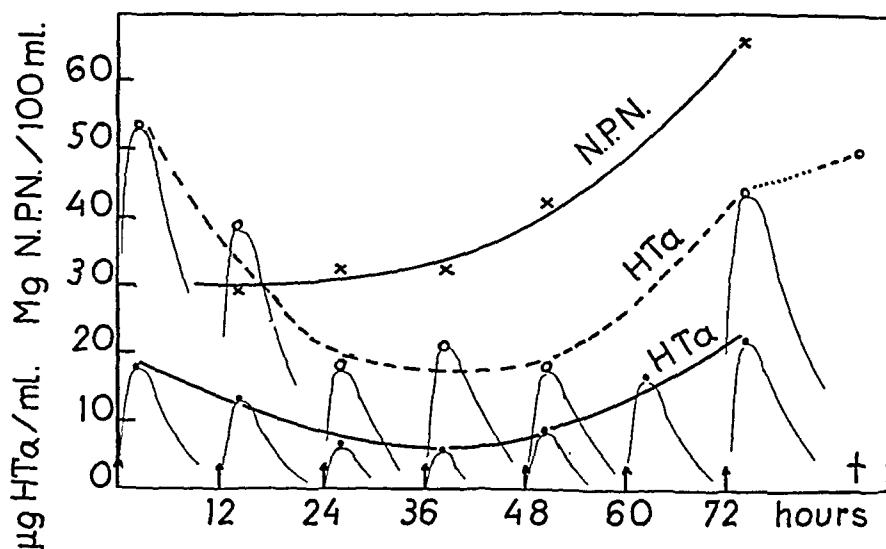


Figure 2.

Tannic acid in plasma after repeated injections of 250 mg (dots) and 500 mg (circles) tannin per kg of body-weight. Every tannin injection is marked with an arrow.

Non-protein nitrogen values belonging to the first series are indicated by x.

out being given any subsequent tannic acid injections. When they were killed after 8 days the hemoglobin value was only slightly lower than before the experiment.

The examinations of these animals showed the following:

Table 4.

Subcutaneous injections of 5 % tannic acid after burns. Dose 200—250 mg tannin per kg of body-weight.

Animal number	71	72	73	¹ 74	75	76
Number of injections	8	9	6	8	8	4
Number of days	10	11	6	6	6	3
Time from last inject. to death in hours	<24	<24	0	6	<6	<12
Conc. of HTa in µg/ml plasma at end of exp.	—	(70)	(206)	55	25	—
Burnt area in % of the whole skin	14	12	5.5	8.4	11	7.5
Total quantity of HTa injected in g/kg body-weight	1.65	1.85	1.05	1.65	1.65	0.85
Liver: Central, acinar necrosis s. necrobiosis	0	0	0	(+)	(+)	0
Kidneys: Degenerative changes in tubular epithelium	0	0	0	0	0	0

¹ killed.

As repeated subcutaneous injections gradually entail reduced absorption of the tannin, a series of experiments with intravenous long-period infusion of a weak tannic acid solution were also carried out. The results are given in table 5.

Table 5.

Intravenous infusions with tannic acid solutions.

Animal number	65	66	67	68	78
HTa in infusion fluid	1.2 %	1.2 %	0.5 %	0.5 %	0.5 %
Infusion time in hours	6	6.5	19	3	28
Time from end of infusion to death in hours	17.5	18×24	2.5	16×24	<16
Conc. of HTa in $\mu\text{g/ml}$ plasma at end of exper.	0	0	0	0	1.3
Total quantity of THa infused in g/kg body- weight	0.265	0.134	0.161	0.265	0.330
Liver: Central, acinar necrosis s. necrobiosis	0	0	0	0	0
Kidneys: Degenerative changes in tubular epithelium	0	0	0	0	0

Microscopic examination of the organs showed no changes, neither in the liver nor in any other organ.

Discussion.

The method worked out for the determination of tannic acid in the blood proved to be fully satisfactory. Blood analyses after subcutaneous injections of tannic acid in rabbits showed a concentration of tannic acid that increased with the amount of injected tannic acid.

Injuries to the parenchyma of the liver localized to the central parts of the acini appeared fairly constantly if large amounts of tannic acid were injected (≥ 2.25 g per kg of body-weight).

No attempts to measure the concentration of tannic acid in the blood or eventually to produce liver-necroses after tannic acid treatment of burns on animals were carried out, as it is impossible to produce in animals the typical burn of the 2nd degree, and one always produces a coagulation necrosis with strongly changed circulation.

As suspicions concerning the toxicity of the tannic acid have arisen in the course of the clinical treatment of burns, it is desirable to assess the results obtained from the experiments on animals from a clinical point of view.

One must then bear in mind that the amounts of tannic acid

that according to the animal experiments would be required to give rise to liver-necroses are many times greater than those used for the tannic acid treatment of even very extensive burns. On the other hand, it may be assumed that a more intensive and even absorption of tannic acid takes place from a burned surface than from a subcutaneous depôt, and that a liver injured by shock from burns may be more sensitive than a healthy liver, so that even a considerably lower concentration of tannic acid in the blood might be conceived to give rise to necrosis of the liver.

At the Surgical Department of St. Göran's Hospital the tannic acid treatment of burns has not been carried out during the past two years, and it has not been possible to observe liver-necrosis in any of the fatal cases or signs of injury to the liver in the course of extensive burns. This experience agrees well with that given in modern publications: cases of necrosis of the liver are rare since the tannic acid treatment of burns has been discontinued.

Summary.

With the intention of investigating the alleged toxic effects of tannic acid on the liver, experimental studies have been carried out on animals. Typical liver-necroses have arisen if large quantities of tannic acid have been injected (at least 2.25 g per kg body-weight). Attempts to produce similar injuries by means of tannic acid treatment of burned surfaces have not been performed.

The experiments give definite support for the assumption that tannic acid is a toxic substance.

The absorption of the tannic acid has been studied with a micro-method specially worked out for the purpose for the determination of tannic acid in blood plasma.

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Investigations of the Effect of Lactic Acid on the Metabolism of Calcium and Phosphorus.

By

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1. Effect of intestinal production of lactic acid on bone formation.

In previous papers MØLLGAARD and collaborators (1946) have shown, that organic oxyacids and particularly tartaric and lactic acid have a very pronounced effect, increasing the absorption of calcium and phosphate from the intestine of the pig. It was further found possible to enlarge the production of lactic acid in the intestine by feeding pigs skim milk cultures of a lactobacillus, that will multiply in the intestines of these animals. This fact established, two large-scale experiments were set up with 40 pigs in order to investigate, whether these animals would grow better and show an improved bone formation, when fed skim milk inoculated with this culture in addition to an ordinary ration containing phytic acid phosphorus in an amount of 60—66 % of the total phosphorus of the ration.

Two groups of equally conditioned young pigs were composed for each experiment, ten animals in each group. The rations of the compared groups were equal except for the skim milk. The one group ("normal") was fed skim milk acidified with the ordinary streptococcus lactis commonly in use in the dairies of Denmark. The other group («acid») was fed the same amount of skim milk acidified with the lactobacillus mentioned. The results of the trial

Table 1.

Effect of feeding skim milk acidified with streptococcus lactis and with a lactobacillus multiplying in the intestines of pigs.

	First "Nor- mal" strepto- coccus	Experi- ment "Acid" Lacto- bacillus	Second "Nor- mal" strepto- coccus	Experi- ment "Acid" Lacto- bacillus
Live weight at begin. of exp. kg	17.1	17.2	19.2	19.3
Live weight at end of exp. kg.	88.8	91.2	88.1	89.9
Feed units pro pig pro day	2.06	2.17	2.09	2.23
Daily gain pro pig pro day g.	613	657	603	641
Feed units pro kg. gained	3.36	3.30	3.46	3.48

with respect to growth appear from Fig. 1 and 2 and from the table 1.

It is seen from the growth curves and from the table, that the groups fed the lactobacillus, grow distinctly better than the "normal" groups, particularly in the younger ages. The amounts of feed units used pro kg. gain are the same in the compared groups, meaning that the only effect with respect to growth is, that the animals of the "acid" groups reach their final weight in shorter time than the normals and eat correspondingly more. However in general condition of health the "acid" groups were distinctly superior to the normals. Particularly their skin was remarkably fine, white and spotless.

After the slaughter an humerus bone was taken from each pig. The bones were sawn through the length and the marrow cautiously washed out with hot water. The result of inspection was very evident and remarkable. The figures 3 and 4 show the most typical and the least typical bone amongst the "normals" and the "acids" of the first experiment. It is easily seen, that the bones from the "acid" group are considerably stronger than the bones from the normal. Their compacta is thicker and their network exceedingly more developed, the medullary room conspicuously reduced. This difference, indicating a superior bone formation in the "acid" groups, was a general mark with all compared animals of the first experiment.

In the second experiment samples of the contents of the small intestine were taken from 7 pigs in each group and inoculated on skim milk. In two of the "normals" the bones showed the same picture as in the "acids", and from the intestines of these

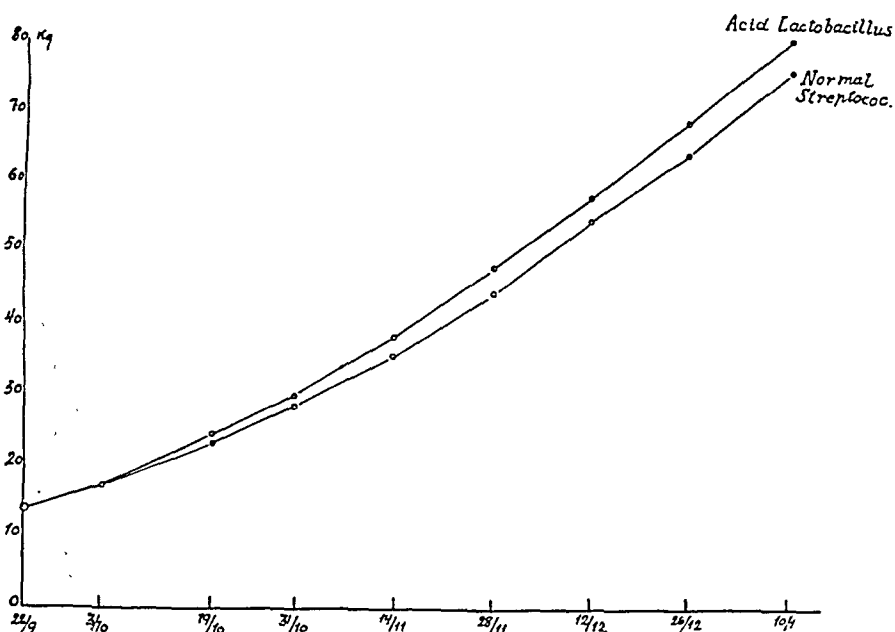


Fig. 1. Growth curves of pigs fed skim milk acidified with streptococcus and lactobacillus. The ordinates represent group averages.

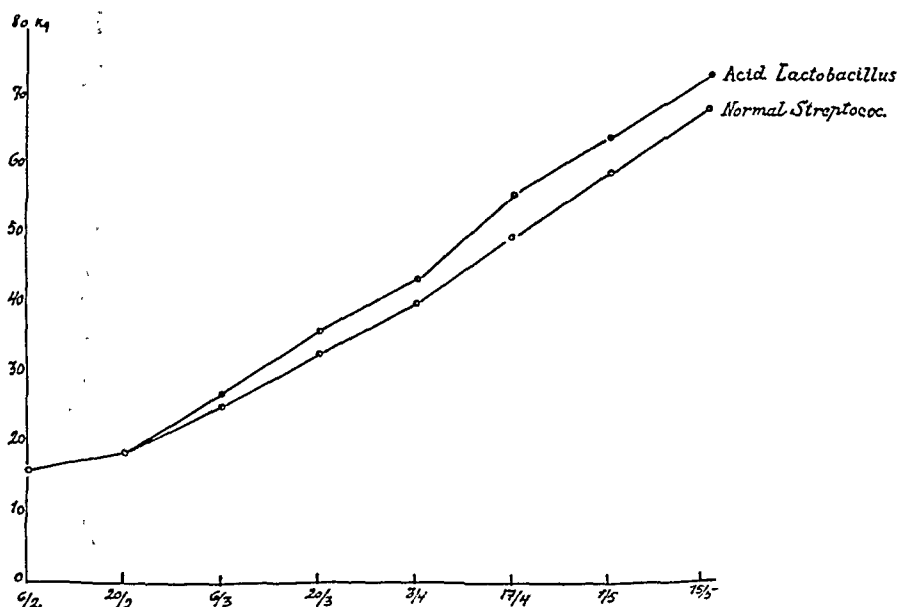


Fig. 2. Growth curves of pigs fed skim milk acidified with streptococcus and lactobacillus. The ordinates represent group averages.

two animals a lactobacillus was cultivated, that in every respect resembled the organism used for acidifying the skim milk in the "acid" groups. In the other "normals" no such bacillus was found. As it would be expected, some pigs are evidently naturally infected with this microorganism. Of the "acids" six gave a culture of the lactobacillus, one none, and in this one animal the humerus bone showed "normal" condition. In all others the bones showed the typical superior bone formation.

One half of each bone in the two groups of the second experiment was dissolved in hydrochloric acid and the average contents of Ca and P determined with the following results, showing

	Normal group	Acid group
Ca %	16.37	17.13
P %	7.98	8.25
Hydroxyapatite %	41.09	43.00

that the mineral contents are higher in the acid than in the normal group.

All these experiments seem to prove beyond reasonable doubt that the beneficial effect of lactic acid on the absorption of calcium and phosphate is of real biological importance, *bone formation being distinctly superior in animals with well developed lactic acid fermentation in the intestines.*

This result is probably of very far reaching importance to our understanding of the mechanism of absorption of calcium and phosphate, presenting a reasonable explanation of some otherwise difficultly understandable experimental evidences found in MÖLLGAARD's laboratory.

2. Absorption of calcium and phosphate with neutral and alkaline reaction in the intestines.

In hitherto unpublished experiments carried out in MÖLLGAARD's laboratory, HAGENS found that by feeding large amounts of sodium hydrocarbonate (150 gram pro day) to normal pigs, he was able to shift the reaction in the jejunum and ileum in neutral direction. The variation was, however, small and determinations of the absorption quotients for Ca and P delivered uncertain results, the quotients being in some cases entirely unaffected, in others significantly depressed. Feeding large amounts of magnesiumtrisilikate and magnesiumoxyde (100 gram pro day) to normal pigs and to pigs with partial resection of the fundus BORCH-MADSEN (1946) got similar results. However, on pigs made completely

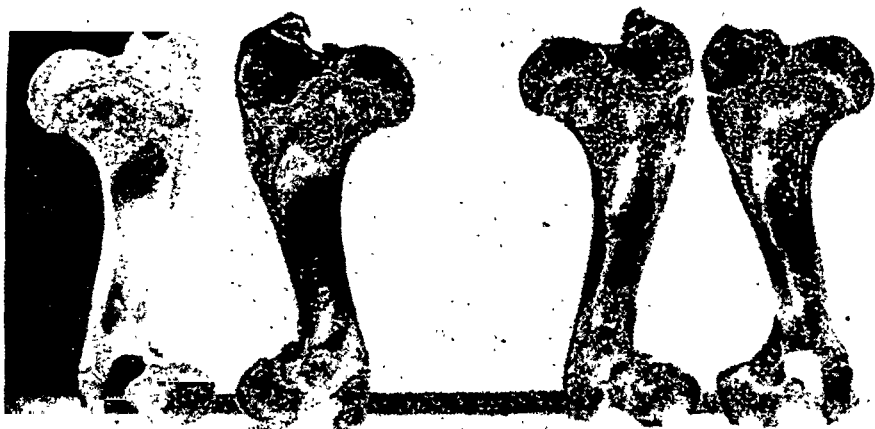


Fig. 3. Humerus bones of pigs fed skim milk acidified with streptococcus. Ordinary bone formation in growing pigs in Denmark.

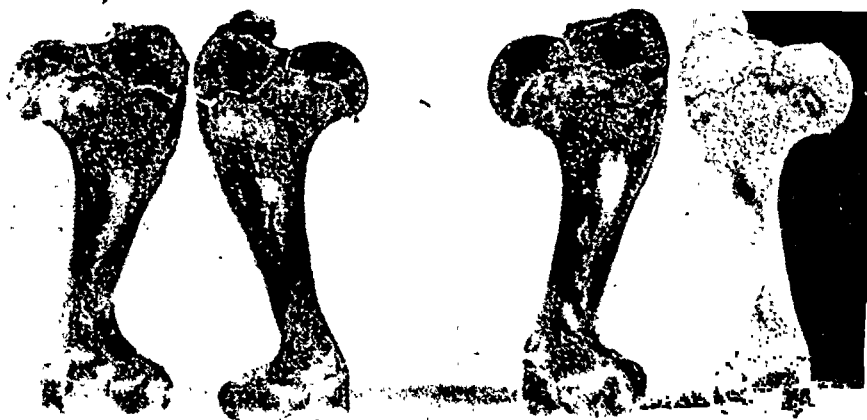


Fig. 4. Humerus bones of pigs fed skim milk acidified with lactobacillus that multiplies in the intestine of these animals. Superior bone formation.

Table 2.

Effect of feeding large amounts of magnesium trisilikate and magnesiumoxyde to complete achylic pig. (Borch-Madsen).

Pig. No	Experiment No.	Food	Ca absorbed %	P absorbed %	Live weight kg
11	1	Normal	31.6	33.6	42
151	1	Normal	26.4	30.3	43
Mean			29.0	31.9	42.5
11	2	Alkaline	21.0	12.5	57
151	2	Alkaline	28.8	18.8	57
Mean			25.4	15.7	57
11	3	Normal	32.6	37.3	88
151	3	Normal	35.6	30.5	85
Mean			34.1	33.9	86.5
11	4	Alkaline	31.9	17.8	93
151	4	Alkaline	20.1	15.7	92
Mean			26.0	16.7	92.5
21	1	Normal	30.5	28.1	70
31	1	Normal	29.5	32.0	66
Mean			30.0	30.1	68
21	2	Alkaline	9.3	8.6	74
31	2	Alkaline	26.9	21.4	73

achylic and responseless to histamine by resection of the whole fundus of the ventricle he was able to effect a very serious displacement of the reaction in duodenum by feeding the trisilikate and oxyde in the amounts mentioned. The pH-values in duodenum went up to 9.0—9.5 and under these conditions, when the reaction in the entire small intestine must have been alkaline, he found a considerable decrease of the absorption quotients for Ca and P. This is shown in table 2. The absolute supply of Ca and P in the compared groups has been very approximately the same. No addition of fat or of vitamin D has been given to any of the animals.

Three results appear evidently from this table:

1) The absorption of Ca is significantly depressed by alkaline reaction in the intestines but has still considerable dimensions except in one pig.

2) The absorption of P is very seriously decreased and much more than the absorption of Ca except in one pig.

3) The decreasing effect of the alkaline reaction is not uniform in all animals, one pig showing a much greater fall of the absorption quotients of both Ca and P.

With respect to absorption of Ca, this means, that under certain conditions Ca may be absorbed even with *alkaline* reaction in the intestines under others (pig 21₂) the absorption drops to an

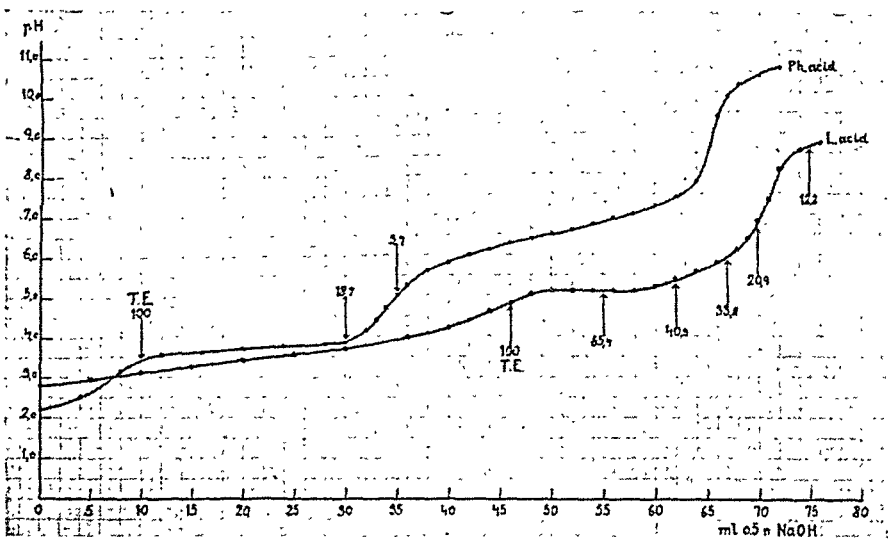


Fig. 5. Titration curves for calciumphosphate dissolved in phosphoric acid and in lactic acid. T. E. means Tyndall effect that is beginning of precipitation. Numbers indicate percentages of Ca still in solution.

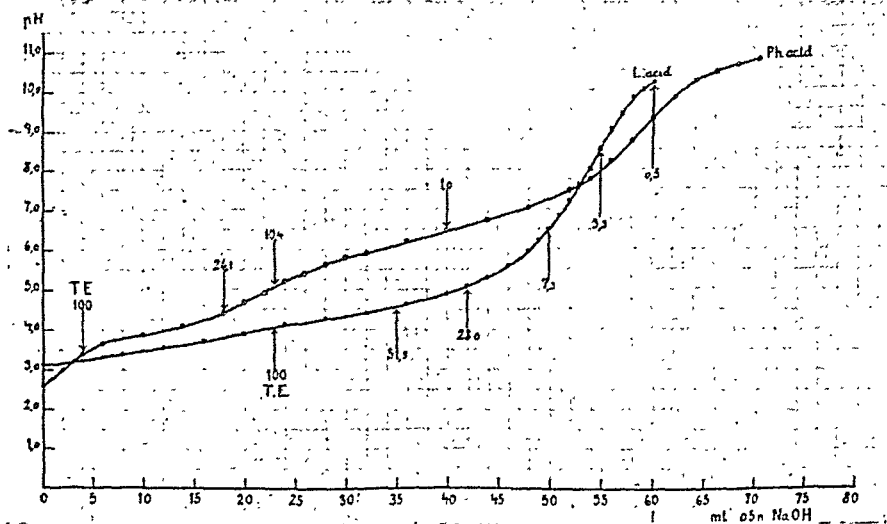


Fig. 6. Titration curves for calciumphytate dissolved in phosphoric acid and lactic acid. T. E. means Tyndall effect. Numbers indicate percentages of Ca still in solution.

Table 3.

Absorption of Ca and P when 80 % and 44 % respectively is given directly into the intestines.

Pig No.	Experiment No.	Minerals given in	Ca absorption %		P absorption %	
43	VIII ₁	Duodenum	24.1	20.9	35.	31.7
33	VIII ₂	Duodenum	17.7		28.3	
43	VIII ₂	Jejunum	33.9	27.1	41.4	36.9
43	VIII ₃	Ileum	28.0		39.7	
33	VIII ₃	Ileum	26.1		34.1	
43 and 33 mean of four experiments		Mouth	38.7		43.0	

unsignificant value. Consequently some animals must contain factors promoting Ca absorption with alkaline reaction in their intestines while such factors are lacking in other animals. With respect to P absorption it means, that an alkaline reaction is more liable to depress the absorption of phosphate than that of calcium, very probably because the phytase of the food is entirely inactivated by a pH of 9, so that the phytic acid passes the small intestine unaltered.

Lastly the results in table 2 indicate, that the absorption of Ca and P goes parallel under the influence of the factors promoting absorption with alkaline reaction.

The reliability of these conclusions has been sustained by experiments of HAGENS (1943). He measured the absorption of Ca and P, when a mixture of CaHPO_4 and CaCO_3 was introduced directly into the intestine of the pig through tubes inserted in duodenum, jejunum and ileum. By this way 80 % of the Ca contents and 44 % of the P contents of the food did not pass the ventricle. The results appear on table 3.

From these experiments the following conclusions may be drawn:

1) The absorption of Ca and phosphate is decreased, when the minerals are withdrawn from the ionizing effect of the acid reaction in the ventricle.

2) By direct introduction in the intestine, the absorption is greatest in jejunum with pH of 5—6, but is still considerable in ileum with pH 6.5 to 7.5.

From the totality of experiments described in this chapter it seems justified to conclude, that the absorption of calcium and

phosphate is largest when their salts are passing the parts of the intestinal canal where they may be ionized to Ca^{++} and H_2PO_4^- , but that a considerable absorption still takes place under such conditions of reaction, where these ions cannot exist, that means where CaHP_4 should be precipitated. The intestines must therefore contain means effecting Ca and phosphate absorption within the higher ranges of pH.

3. Nature of agents promoting absorption of Ca and P within higher ranges of pH.

In order to be absorbed a substance must be dissolved under the conditions prevailing in the intestines. More precisely expressed this means that it must be water soluble at the pH the ion pressure and the partial concentrations of dissociated and undissociated compounds existing at each time in the different parts of the small intestines.

If conditions are such, that calcium and phosphate cannot be dissolved as the ions Ca^{++} and H_2PO_4^- , the possibility of absorption seems to depend entirely on the presence in the intestine of substances, forming complex calcium and phosphate compounds soluble in water under these conditions. In that case two ways of absorption are possible:

1) The soluble complex may be absorbed itself and split by enzymic cleavage in the mucosa of the intestine.

2) The soluble complex may still dissociate calcium and phosphate ions at a rate, corresponding to the absorption velocity of these ions, which then may be continuously absorbed, the equilibrium: C ion · C complex former \rightleftharpoons C complex · k being steadily readjusted as absorption goes on.

The first principle is known from the absorption mechanism of high molecular fatty acids, but except for glucosephosphate we have no evidence of its working in absorption of calcium and phosphate.

For the second principle there seems to be good evidence in the experiments with oxyacids mentioned in the first chapter of this paper. The results with tartaric and lactic acid cannot very well be explained otherwise than being of this character. Under the ordinary conditions of feeding the lactic acid is very probably the sole oxyacid formed by enzymatic processes in the intestinal canal, but it may be that oxyacids of the type of bile acids, preferably the desoxycholanic acid are able to promote absorption of calcium and phosphate by forming complex compounds too. It has long

been suggested, that the bile has an effect on calcium absorption. That this is a fact appears from table 4, that contains the principal results of experiments carried out by LUND (1945) on pigs before and after blocking of the bile duct to the duodenum and opening the gall bladder by inserting a tube.

Table 4.

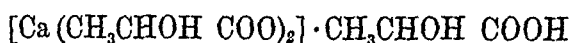
Absorption quotients before and after blocking of the bile duct.

	Protein	Fat	Ca	P	Vit. D. added	No of exp.
Before blocking	80	58	46	50	2,400 I. E.	12
Before blocking	75	65	46	46	1,200 I. E.	8
After blocking	77	—	33	36	2,400 I. E.	3
After blocking	78	11	30	39	1,200 I. E.	9

4. Nature of the complex calciumphosphate oxyacid compound and its behaviour with increasing values of pH.

From the experiments of MÖLLGAARD and collaborators on the effect of oxyacids it appeared, that these substances increase the absorption of Ca and P approximately to the same amounts, indicating a parallelism in absorption of these elements. This suggests that both calcium and phosphate enter the complex.

From technical chemistry it is well known, that there exists a calcium lactate of the following composition:



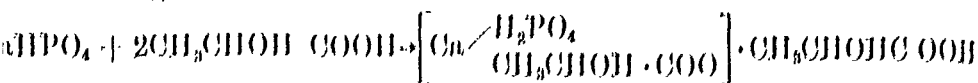
That one of the two lactate ions directly combined with calcium could be replaced by a dihydrophosphate ion forming a soluble calciumphospholactate is a probable suggestion. To investigate this possibility a great surplus of CaHPO_4 was shaken with a solution of lactic acid for 4 hours at a temperature of 40° . Increasing time made no difference of results. After filtering the contents of Ca, P and lactic acid was determined quantitatively. The results of two experiments appears from table 5.

Table 5.

Dissolution of CaHPO_4 in lactic acid at 40° .

	First experiment	Second experiment	
Ca	0.277	0.293	g atom pro Liter
P	0.225	0.252	g atom pro liter
Lactic acid	0.555	0.609	mol pro liter
Lactic acid: Ca	2.00	2.08	
Ca: P in the original phosphate: 1.1			

The table shows that very approximately 1 gram atom Ca dissolves in 2 mol lactic acid at the concentrations indicated. This result can hardly be explained as simple ionizing of the phosphate by taking up a hydrogen ion at the acid reaction prevailing in the solution (pH ca. 3). It is however quite naturally explained by the following reaction:

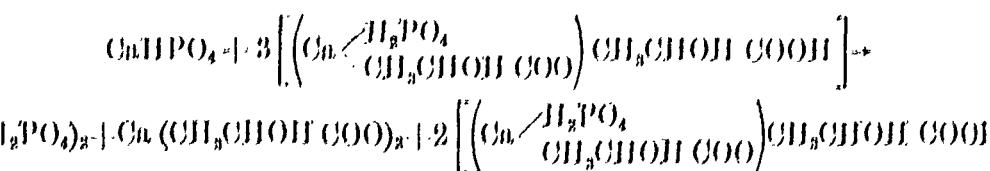


Because the relation C : P is a little bit over 1 as well in the original phosphate as in the solution, small amounts of $\text{Ca}(\text{CH}_3\text{CHOH}(\text{COO}))_2$ must be present too.

After diluting the solution to approximately the double volume and shaking with CaHPO_4 for another 4 hours period the following concentrations were found:

Ca	0.193 g atom pro liter
P	0.160 g atom pro liter lactic acid; Ca : P = 1.54
Lactic acid:	0.297 mol pro liter

In more diluted solutions the quotient Ca: lactic acid consequently appears to be 2:3, which means, that the complex calciumphospholactate partially dissociates according to the following reaction:



It is easily seen, that these experiments strongly sustain the idea, that lactic acid in the intestines forms a complex calcium-phospholactate, and that this complex slowly dissociates to Ca^{++} , H_2PO_4^- and $\text{CH}_3\text{CHOH}(\text{COO})^-$ as the concentration of these ions decreases at an approximately constant pH value.

After this the behaviour of the complex with increasing values of pH was investigated in the following way. In a solution of lactic acid containing 0.7 mol pro liter was dissolved 0.26 mol CaHPO_4 . The analyses of the solution gave 0.263 gram atom Ca and 0.236 gram atom P. This solution was titrated by a 0.5 molar solution of NaOH, the pH being measured at the glass electrode. Exactly the same experiment was made with the same concentration of CaHPO_4 dissolved in phosphoric acid in a concentration equivalent

to the lactate ion concentration of the first solution, the phosphoric acid being considered trivalent. The results of titration appear from Fig. 5.

It appears evident from the curves, that when the calciumphosphate is dissolved in phosphoric acid, the precipitation begins at a pH about 3.7 and is almost complete at pH 5.0. When however the phosphate is dissolved in lactic acid the precipitation begins at pH 4.8 and is not completed even at pH 8.7 approximately 12 % still being in solution. It is further evident from the flatness of the curve, that the release of calcium and phosphate ions goes on very slowly. These results cannot very well be otherwise explained, than that the solution contains a complex, that is completely stable until pH 5 and hereafter slowly dissociates as the pH increases. Consequently the titration curve for the lactic acid solution reflects all the theoretical assumptions needed to explain the effect of oxyacids, the absorption of calcium and phosphate at neutral and alkaline reaction and the parallels between the absorption of these minerals. As furthermore the complex is completely stable until pH 5 it appears that the absorption by means of complex formation begins just at the reaction, when hydrophosphate ions are formed in such amounts, that the absorption of both minerals otherwise might be seriously depressed. The analyses of the precipitates have shown that until the point of neutrality they consist of CaHPO_4 .

Similar results were found, when calcium phytate is dissolved in phosphoric acid and lactic acid. The titration curves are seen on Fig. 6, from which it appears, that the precipitation begins in the first case at pH 3.4 in the other at pH 4.1 and that the lactic acid solution at pH 5.1 contains 3 times as much Ca as the phosphoric acid solution. At 6.5 the contents are 8 : 1. In this case too the curve seems flat enough to permit absorption velocity in the intestine to equalize dissociation velocity of the complex.

Summary.

1. Strong lactic acid fermentation in the intestines of the pig effect an increase of the absorption of calcium and phosphate and by that way a superior bone formation. It further promotes health and growth of young pigs considerably.

2. The absorption of calcium and phosphate from the intestine takes place partially as a simple absorption of the ions Ca^{++}

and H_2PO_4^- and partially by the intermediate formation of a complex calciumphospholactate, that is stable until pH 5, but with increasing pH dissociates so slowly, that the absorption velocity may be considered equal to the velocity of release of Ca^{++} and H_2PO_4^- .

3. The bile acids probably exert a similar effect on absorption as the lactic acid.

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A Simple Permeability Experiment for Class Work and Demonstration Purposes.

By

RUNAR COLLANDER and TEUVO ÄYRÄPÄÄ.

Received 19 May 1947.

For a demonstration of the selective permeability of living cells it is, in most cases, necessary to use a microscope. It is, however, our purpose here to describe a very simple experiment — or rather a series of simple experiments — for which no other apparatus than a test-tube is needed. On the same time these experiments are very impressive. They are, therefore, adapted both to class work, even for beginners, and also to demonstration purposes.

Suspend a teaspoonful of baker's yeast in about 50 ml. of a 0.5—1 per cent. sodium carbonate solution. Fill a test-tube to about $\frac{1}{3}$ with the cell suspension and add an almost equal volume of a 0.02 per cent. aqueous solution of neutral red. Mix the contents of the test-tube thoroughly. Due to the alkaline reaction of the suspension fluid the colour of the neutral red turns yellow at first, but in the very next moment the reverse colour change begins so that the suspension during the lapse of about one minute turns successively orange-red, brick-red, and ultimately pink-red. When the last-mentioned colour has been reached, it remains unchanged, or at least nearly so, for hours and even for days. With such a pink coloured cell suspension the following tests are made: — (1) One portion of the cell suspension is filtered: the yeast is retained as a deeply stained mass on the filter while the filtrate shows colourless. — (2) To another portion of the suspension, add some 0.01 normal sodium hydroxide or potassium hydroxide solution: no colour change occurs. — (3) To a third

portion, add some 0.01 normal ammonia solution: the colour of the suspension turns yellow at once. — (4) Heat a portion of the suspension over the flame of a gas-burner: its colour changes yellow.

If performed on a larger scale, using glass cylinders of 100—200 ml. capacity, these experiments can be demonstrated also to a large audience.

From these colour changes the following conclusions may be drawn: — (a) The brownish-yellow neutral red base obviously penetrates very rapidly into the yeast cells, for already after the lapse of about one minute the dye is almost entirely accumulated in the yeast cells, the volume of which amounts only to about 1—2 per cent. of the total volume of the suspension. The great penetration power of the dye base is, no doubt, due to its considerable lipoid solubility (cfr. COLLANDER, LÖNEGREN and ARHIMO 1943) while the capacity of the cells to hold back the dye taken up by them is explained by the fact that the base, after it has entered the cells, is immediately bound by some acid constituents of the cells as can be seen from the fact that its yellow colour changes to pink. (The major part of the dye taken up by the cells seems to accumulate in the vacuoles; cfr. BRANDT 1941.) — (b) The fact that the pink-red colour persists in spite of the distinctly alkaline reaction of the extracellular sodium carbonate solution shows that the cells, while very permeable to the large molecules of the neutral red base, are at the same time practically impermeable to the much smaller ions of the sodium carbonate solution. As even an addition of dilute sodium hydroxide or potassium hydroxide solution has no perceptible effect on the colour of the suspension it is clear that the cells are highly impervious also to these substances. — (c) On the other hand, the sudden colour change occurring on addition of ammonia proves the extremely rapid penetration of this substance. Also alkyl monoamines, and many alcaloids too, act in a manner similar to ammonia, while alkyl diamines cause but a much slower change of the intracellular pH. — (d) The colour change brought about by heating the solution shows that the impermeability of the cells to the alkali disappears when the cells die.

Finally it may be pointed out that yeast stained with neutral red yields a favourable material, not only for such crude demonstration experiments as described above, but also for quantitative determinations of the penetration power of different bases. By

using a mixing device like that described by HARTRIDGE and ROUGHTON (1927) even the very rapid progress of the colour changes brought about by the most rapidly penetrating bases can be satisfactorily followed. The results arrived at in such quantitative experiments will be described elsewhere. Suffice it, here, to say that our observations confirm the older ones of POIJÄRVI (1928) on other plant cells in that the lipoid solubility is found also in this case to be the chief factor determining the penetrating power of the different bases while on the other hand the smallest base molecules (especially those of ammonia and methylamine) penetrate so fast that it seems difficult to assume that the plasma membrane of the yeast cells is a homogeneous lipoid membrane. At any rate, our experience indicates that the permeability of yeast cells, hitherto so incompletely known (cfr. ØRSKOV 1945), agrees at least in principle very closely with that of most other plant cells.

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Studies on the Action of Dibenzyl- β - Chloroethylamine.

By

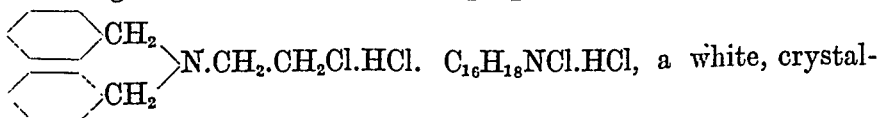
GUNNAR BIÖRCK.

Received 21 May 1947.

Although there is fair evidence, that increased action of the sympathetic nervous system can explain some of the manifestations in coronary, hypertensive and peripheral vascular disease yet very little (except for surgical interventions) has been done in the way of specific treatment thereof. This is apparently due to the fact, that the drugs with sympatholytic action (for example ergotamine) in therapeutic doses exert an influence which is not always predictable and is often accompanied by unpleasant or even toxic manifestations. In man, for example, there may be at the same time an inhibitory effect on the sympathetic, while the direct action of the drug on the smooth muscles of the vessels may cause vascular constriction.

When therefore it was reported by NICKERSON and GOODMAN (1946) that a new sympatholytic drug had been synthesized, which was claimed to exert a more lasting effect than ergotamine, it was obvious that an investigation should be made of its applicability in man in the treatment of those conditions where an inhibition of the excitatory sympathetic activity would be desirable.

The Astra Company, Södertälje, Sweden, was kind enough to synthesize the dibenzyl- β -chloroethylamine-HCl for the purpose of such an investigation. The compound in question has the following chemical formula and properties:



line powder with a molecular weight of 296, melting point 190°C , easily soluble in water and alcohol. (An aqueous solution has been used in this study.)

Comparison with "Dibenamine", manufactured by the Givaudan-Delawanna Company, New York, N. Y., used in the above mentioned report and kindly put at my disposal by Doctor MARK NICKERSON, has shown, that the preparations are chemically identical.

The experimental studies have been planned from the point of view of clinical trials later on. They have, accordingly, comprised investigations concerning the effect on blood pressure, respiration and heart action (ECG) in the anesthetized animal (cat, rabbit), the development of anaphylaxis (rabbit, guinea-pig) and the eventual toxic actions on heart, liver, kidney and brain (rat, rabbit) when given repeatedly. Also the effect on metabolism (rat) and the ability of the drug to prevent a fatal outcome after a lethal dose of adrenaline subcutaneously (rat) were investigated.

The results of these experiments have not been encouraging enough to prompt a clinical trial as yet, although it is known that such an investigation is being carried out in the U. S. A. The compound, however, may prove quite useful for purposes in connection with animal experimentation, and the results encountered during this study are therefore being reported.

I. Anaphylaxis and histo-toxic reactions in the course of repeated administration of dibenzyl- β -chloroethylamine.

A. Anaphylaxis. Experiments with 3 rabbits and 2 guinea-pigs, all of which got at least 5 injections each of 20 mg dibenzyl- β -chloroethylamine (or more) in the course of one to two months did not reveal any sign of anaphylactic reaction.¹ The injections were given intramuscularly in the thigh. None of the animals showed any local tissue reaction or general reaction immediately or later on. The guinea-pigs showed normal increase in body weight during the time of observation. It is therefore concluded that the substance has not shown any tendency to induce anaphylactic reactions in the rabbit or the guinea-pig. Later studies on rabbits and rats have confirmed this observation.

B. Histo-toxic reactions. Four rats and two rabbits were injected daily with increasing doses of dibenzyl- β -chloroethyl-

¹ These experiments were performed through kind assistance of Dr. PER HEDLUND, State Bact. Laboratory.

amine for between one and two and a half months. Control animals were studied together with the injected ones. All the injections were given intramuscularly in the thigh with the exceptions of intravenous doses of 8 mg/kg for the first five days in the rabbits and 50 mg/kg in one of the rats following two months of intramuscular injections.

The intravenous injections in the ears of the rabbits had to be discontinued on account of local thrombosis. No local damage was noted from the intramuscular injections. The rat, which got intravenous injections, died after one week in convulsions, five minutes after an injection. One of the rabbits died from coccidiosis after $1\frac{1}{2}$ months.

The rabbits received for the first 5 weeks 15 mg/kg daily and later on 50 mg/kg daily. The four rats were given for the first 5 weeks respectively, 5, 12, 20 and 40 mg/kg daily. The rat which received 40 mg/kg was killed after the 5 weeks, and, as no pathological tissue changes were seen, the doses for the other rats were increased to respectively 60 and 200 mg/kg. In one of these latter rats intravenous injections of 50 mg/kg were given for some days, as mentioned above. In none of the animals, which died spontaneously or were killed, were any microscopic changes¹ seen in the brain, heart, liver or kidney (apart from coccidiosis in one rabbit). All the animals showed a normal weight or even increase of weight during the experiment. It seems therefore safe to conclude that the administration of dibenzyl- β -chloroethylamine even in massive doses intramuscularly for a long time does not cause organic damage to the brain, heart, liver or kidney in the rabbit or rat.

II. Effect on blood pressure, respiration, heart action and response to adrenergic stimuli in the anesthetized animal.

13 experiments on rabbits and 18 on cats have been performed in order to study the effect on the blood pressure, respiration, heart action and response to adrenergic stimuli in anesthetized animals after injection of dibenzyl- β -chloroethylamine. The blood pressure was recorded by means of a Hg-manometer connected with a cannula in the left carotid artery and the intravenous injections were made in one of the femoral veins. The respiration was studied in some experiments by means of the body plethysmograph designed by VON EULER and LILJESTRAND (1936). The heart action was registered by an electro-

¹ Med. Dr. F. WAHLGREN has kindly performed the microscopical examination of the organs.

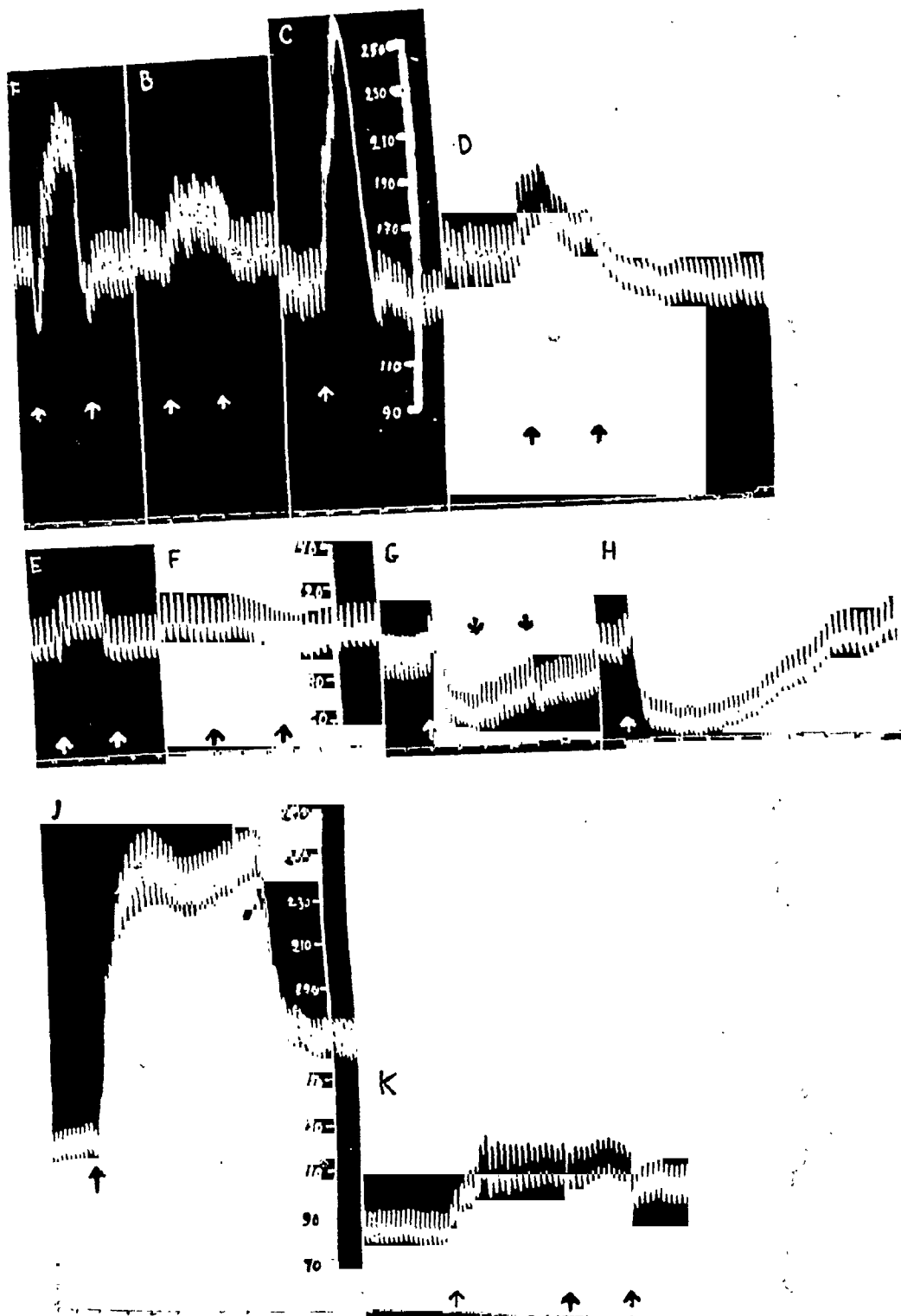


Fig. 1.

cardiograph, "Elmqvist Junior", kindly placed at my disposal by The Jährns Electrical Co. The animals either breathed ordinary air or were given gas mixtures from bags connected with Müller valves.

The paper by NICKERSON and GOODMAN (1946) regarding the physiological properties of the dibenzyl- β -chloroethylamine compounds points out that the preparations have been found to block and reverse excitatory adrenergic responses in mice, rats, rabbits, cats, dogs and humans. They are said to be effective by all routes, although local necrosis may result from subcutaneous and intraperitoneal administration. In a personal communication NICKERSON has later admitted that reversal of adrenergic response is not demonstrated in rabbits.

Both intravenous and intramuscular injections have been used in the present investigation. Intramuscular injections of 20 mg/kg up to 100 mg/kg of a fresh solution of equal amounts of dibenzyl- β -chloroethylamine-hydrochloride (20 mg/ml) and Ringer's solution in cats have as a rule shown no significant effects on blood pressure or the response to adrenergic stimuli such as carotid sinus occlusion or injection of adrenaline within 1—1½ hours. More striking effects were obtained with "Dibenamine" in alco-

The effect on blood pressure of slowly performed intravenous injection of dibenzyl- β -chloroethylamine. Cat, chloralose anesthesia.

- A. The effect of carotid sinus occlusion in the animal before the experiment. The arrows denote clamping of the carotid artery and release of the clamp. Time marking, half minutes.
- B. The effect of breathing a gas mixture containing 7.1 % oxygen. The arrows denote the beginning and the end of such breathing.
- C. The effect of intravenous injection of 1 ml adrenaline 1: 40,000. The arrow shows the beginning of the injection. The scale shows the pressure in mm. Hg.
- D. Injection of dibenzyl- β -chloroethylamine, 10 mg/kg slowly intravenously. Arrows denote beginning and end of the injection.
- E. The effect of carotid sinus occlusion 20 minutes after the injection. Arrows as in A.
- F. The effect of breathing the oxygen-poor gas, 18 minutes after the injection. Arrows as in B.
- G. Intravenous injection of ½ mg. of KCN 40 minutes after the injection of dibenzyl- β -chloroethylamine. The first arrow denotes the injection of KCN, the second and third respectively clamping of the carotid artery and the release of the clamp.
- H. Injection of 1 ml of adrenaline 1: 40,000 intravenously 30 minutes after the injection of dibenzyl- β -chloroethylamine. The arrow shows the beginning of the injection.
- J. The effect of intravenous injection of posterior pituitary hormone (Hypadrin, Astra 1 cc. = 10 I. E.). The first arrow denotes that injection. At the second arrow 1 ml. of adrenaline was injected with a resulting fall in blood pressure.
- K. The effect of intravenous injection of 0.25 mg. of ergotamine (Gynergen, Sandoz) 45 minutes after the injection of dibenzyl- β -chloroethylamine. The first arrow denotes the injection of ergotamine, the second and third respectively clamping of the carotid artery and the release of the clamp.

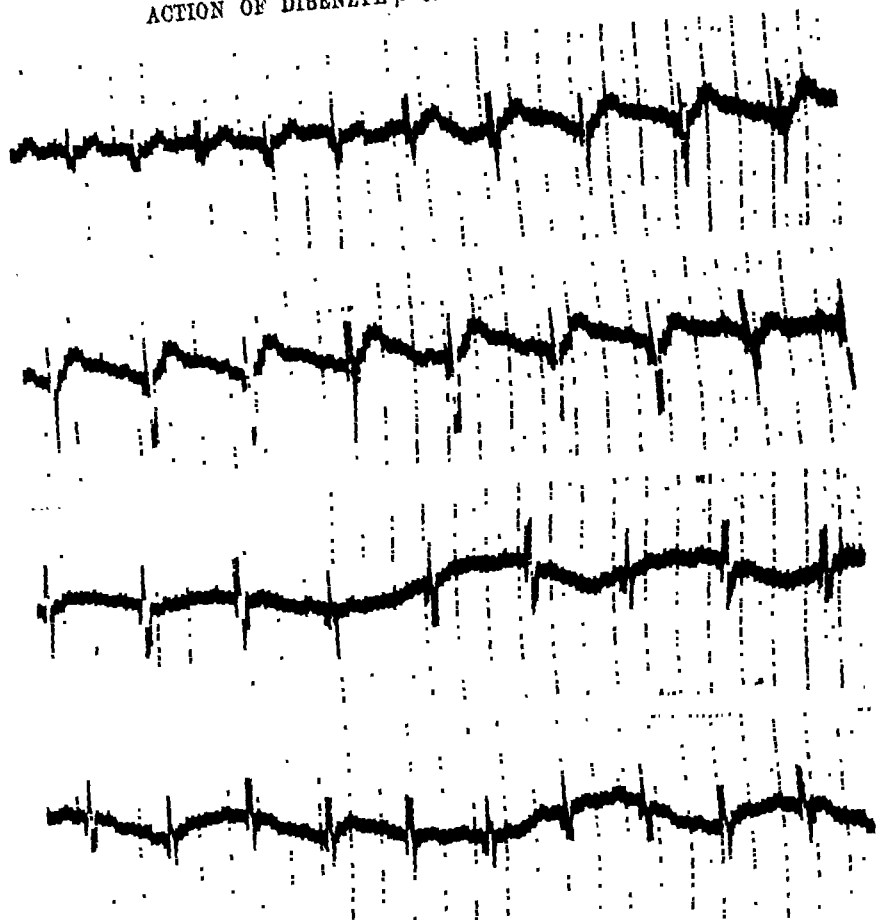


Fig. 2.

Electrocardiogram (lead I) showing the effect on heart rate and other changes on the rapid injection of 1 ml. (8 mg/kg) of dibenzyl- β -chloroethylamine. Rabbit, urethane anesthesia. Upper curve: the change occurring immediately on the injection. The following curves show the sequence of events from about the third second to the fifteenth second. Time marking: $\frac{1}{10}$ second between two thick lines.

holic solution. It is therefore possible that the preparation is more stable and effective in alcoholic solution.

Intravenous injections have revealed a peculiar behaviour of the compound. Whereas injections, performed slowly, are sometimes accompanied by a slight increase in blood pressure, rapid injections of moderate doses invariably result in an immediate fall in blood pressure for $\frac{1}{2}$ —1 minute, after which time the blood pressure returns to its former level or may rise even higher. The former injections — if the amount of the compound is sufficient — after the course of some 5—20 minutes result in blocking of the response to excitatory adrenergic stimuli. The latter injections as a rule do not have this effect. It seems therefore justifiable

to report first on the immediate affect of rapid intravenous injection of dibenzyl- β -chloroethylamine and secondly on the effects of slowly performed injections.

A. Rapid injection.

Small doses, such as 5—10 mg/kg often cause a rise in blood pressure. Larger doses, such as 10—20 mg/kg always give a rapid fall in blood pressure, generally in proportion to the dose used. Doses of about 20 mg/kg are usually lethal, although the animal can in some instances be rescussitated by means of large doses of adrenaline intravenously. Doses of 25 mg/kg or more seem to be invariably lethal in spite of all efforts to rescussitate the animal. It has in some instances been noted, that even smaller doses may prove fatal when given at the peak of the reaction following intravenous adrenaline. This may perhaps be due to a direct effect upon the heart, sensitized by the adrenaline.

The effect on blood pressure is usually of short duration and the initial pressure is usually reached or even exceeded in one minute. Blocking or even reversal of the response to adrenergic stimuli such as injection of adrenaline, occlusion of the carotid arteries or asphyxiation has in some few instances been observed when applied during the phase of the fall in blood pressure. A short while afterwards, however, adrenergic stimuli have generally resulted in significant elevations of blood pressure. In some few instances there was a quantitative reduction of the response, particularly to carotid sinus occlusion. No summation of effects has been demonstrated as long as the individual doses were kept within the above limits.

Higher, non-fatal doses are often accompanied by convulsions, both tonic and clonic in character. The intravenous injection in both anesthetized and non-anesthetized animals was accompanied by a short period of apnoea, followed immediately by hyperventilation as the fall in blood pressure ceased. Electrocardiograms in connection with the experiments show, that the rapid injection is accompanied by a pronounced decrease in heart rate (Fig. 2). At the same time there may be a broadening of the QRS complexes and they assume the picture of a bundle branch block. Higher doses may be followed by further changes such as complete heart block and asystole. The decrease in heart rate is often combined with electrocardiographic signs of coronary insufficiency, possibly caused by the decrease in the venous return

and inefficient coronary circulation. All these changes may be due to unopposed action of the vagus during momentary paralysis of the sympathetic nervous influence.

B. Slow injection.

Before injection the solution of dibenzyl- β -chloroethylamine-hydrochloride (20 mg/ml) was diluted with an equal amount of Ringer's solution. The injection into the femoral vein was usually performed in one or two minutes. With this technique even small doses such as 3.5–4 mg/kg could in some of the experiments both in cat and in rabbit produce a significant blocking of the influence on blood pressure of adrenergic stimuli. The injection itself is often accompanied by a slight increase in blood pressure (Fig. 1 d). The sympatholytic effect may start in the course of few minutes and has usually reached its maximum intensity after 15–30 minutes. It seems as if the effect of such small doses only persists for about one hour, whereas the effect of larger doses persists for a longer time. In one experiment, where the cat was given 15 mg/kg continuous blood pressure recordings revealed an unimpaired effect for at least $10\frac{1}{2}$ hours, after which the experiment had to be discontinued. NICKERSON and GOODMAN (1946) report effects lasting over 2–3 days. Some few animals however have been very resistant to the drug and required larger doses with a longer latent period before any effect was observed.

The blocking effect has been studied by means of the carotid sinus occlusion test (Fig. 1 A), by breathing a gas mixture with 7.1 % oxygen in nitrogen (Fig. 1 B) and by injection of adrenaline (Fig. 1 C). The oxygen deficiency test has been least useful, because with the use of 7.1 % oxygen in nitrogen the general condition of the animal and particularly of the heart action after the injection of dibenzyl- β -chloroethylamine have been so impaired that valid conclusions could not always be drawn. After the injection of dibenzyl- β -chloroethylamine slowly intravenously the normal response to carotid sinus occlusion was abolished (Fig. 1 E), the normal anoxic rise of blood pressure was replaced by a corresponding fall (Fig. 1 F, G) and the injection of adrenaline also resulted in no rise or even a fall in blood pressure (Fig. 1 H). The carotid occlusion test in many cases seemed to give the earliest indication of the sympathetic blocking effect. The adrenaline test gave in rabbits no reversal (*i. e.* no fall in blood pressure after treatment of the animal with dibenzyl- β -chloroethylamine),

which was, however, generally the case in cats. The magnitude of the fall in blood pressure bore little relation to the dose of adrenaline used, while the duration of the effect was on the contrary roughly proportional to the adrenaline dosage. The "reversal" was accompanied by an increase in heart rate. A reversal was also encountered by intravenous injections of benzedrine (phenopromine), which shows that the effect of dibenzyl- β -chloroethylamine is not of the same kind as that of ergotamine, where there is no such reversal.

In the experiments with low doses of dibenzyl- β -chloroethylamine there was some evidence, that large amounts of adrenaline were able to overcome and destroy the blocking effect, whereafter smaller doses could again manifest their normal excitatory effect.

The electrocardiograms have revealed less striking features than in the series with rapid injection. The heart rate is much less influenced by the slow injection. The decrease in heart rate is probably not due to the fall in blood pressure as a moderate slowing of the heart is seen together with even an elevation of blood pressure during the injection. Immediately following the injection there are often observed signs of coronary insufficiency, and in the final stages bundle branch block and bradycardia may develop. In many of the blood pressure curves it is observed that the amplitude of the blood pressure is diminished for some time after the injection of dibenzyl- β -chloroethylamine. This may be another expression of impaired heart function caused by the injection.

The effect of some other drugs in connection with the use of dibenzyl- β -chloroethylamine has also been investigated. Intravenous injection of posterior pituitary hormone during the period of maximal blocking effect of the dibenzyl- β -chloroethylamine resulted promptly in a marked increase in blood pressure (Fig. 1 J). This long-lasting elevation of blood pressure could however be interrupted at any time by the reversal caused by injection of adrenaline (Fig. 1 J). Intravenous injections of $\frac{1}{4}$ — $\frac{1}{2}$ mg ergotamine (Secatotal Astra, Gynergen Sandoz) also maintained a moderate stimulating effect on blood pressure at the height of the sympathetolytic action of the dibenzyl- β -chloroethyl compound. (Fig. 1 K.)

III. Prevention of lethal effects from an overdose of adrenaline.

In the paper by NICKERSON, SMITH and GOODMAN (1946) it is stated that dibenzyl- β -chloroethylamine HCl affords a considerable protection to epinephrine induced cardiac irregularities in dogs sensitized by cyclopropane. This would also be reasonable with regard to the sympatheticolytic action of the compound. A study was therefore performed to investigate, whether the previous injection of dibenzyl- β -chloroethylamine in rats would afford any protection against the injection of lethal doses of adrenaline subcutaneously. It has been stated by NICKERSON and GOODMAN (1946) that the compound is effective also in this species.

Seven white rats weighing between 140—170 g. were injected subcutaneously with 2.5 cc. adrenaline of a 1 % solution (roughly corresponding to 15 mg/kg adrenaline). All of them died from 70—246 minutes after the injection, the average being 150 minutes.

Nine other white rats of the same size were injected first with 10 mg/kg of dibenzyl- β -chloroethylamine and then, after 3—4—1½ hour with 2½ cc. of the same solution of adrenaline. Three of the rats had the dibenzyl- β -chloroethylamine injected intramuscularly, the other six intravenously. One of the rats, which had got the intramuscular injection survived, the other two both died after 45 minutes. None of the intravenously injected rats survived. They died from 60—120 minutes after the injection, the average being 136 minutes. There was no difference in behaviour between the "protected" and "unprotected" rats and they probably all died an "acute heart death".

In another experiment with an anesthetized cat electrocardiograms were taken during the increase in blood pressure due to intravenous injection of adrenaline and during the fall of blood pressure after such an injection when the animal had previously been treated with dibenzyl- β -chloroethylamine. Regardless of the direction of the change in blood pressure there was in both instances a moderate augmentation of the heart rate. Smaller changes in the QRS were however observed in the latter case than in the former. However, there is no evidence from the above mentioned experiments that, with the doses used, dibenzyl- β -chloroethylamine in rats affords any protection against the lethal effect (presumably on the heart function) of large doses of adrenaline.

Discussion.

These experiments have shown, that dibenzyl- β -chloroethylamine is a potent drug acting on the blood pressure mechanism in anesthetized cats and rabbits. Changes in the heart rate and in the electrocardiogram have been noted following administration of the drug and there is also a short period of apnoea at the beginning of intravenous injections, later followed by hyperventilation. With increased dosage clonic and tonic convulsions are noted. Prolonged administration of the drug intramuscularly in rabbits, guinea pigs and rats has caused no demonstrable damage to brain, heart, liver and kidneys, nor has it caused any signs of anaphylaxis. The mode and rate of administration are important. The effect of intramuscular injections seems to be favoured when alcoholic stock solutions are used. Rapid intravenous injections result in an instantaneous drop of blood pressure lasting about one minute not followed by any long-lasting blocking of excitatory adrenergic responses. Slowly given injections, on the other hand, may even cause an increase in blood pressure during the injection, and they result in a blocking of the excitatory adrenergic responses which lasts for many hours. Thus the carotid occlusion test shows a loss of the normal pressor response, breathing of air with low oxygen tension causes a fall in blood pressure instead of an elevation (cat) and injection of adrenaline also brings about a marked fall in blood pressure, "adrenaline reversal" (cat). In rabbits there is no "reversal", but the response to anoxemia and adrenaline is almost completely blocked. Posterior pituitary hormone ("Hypadrin", Astra) and ergotamine ("Gynergen", Sandoz and "Secatotal", Astra), however, give rise to a marked pressor response when injected intravenously after previous treatment of the animal with dibenzyl- β -chloroethylamine in doses sufficient to elicit a typical adrenaline reversal effect (in the cat).

The action of the drug on adrenergic inhibitor responses has not been studied in the present investigation. It is, however, reported by NICKERSON and GOODMAN (1946) that the blocking effect is restricted to the adrenergic excitatory manifestations, whereas inhibitory responses to adrenaline are not affected. This suggests that the drug acts distally in the chain of events, between the adrenaline or the sympathin liberated at the end-organs and the contractile substance itself.

Rapid injections are followed in most cases by transient brady-

cardia and sometimes by broadening of the QRS-complexes, bundle branch block and complete heart block. Signs of coronary insufficiency are also frequently noted. This may be due either to the unopposed action of the vagus nerve during the momentary paralysis of the sympathetic or to the fall in blood pressure and decrease of the venous return or to a direct effect on the heart muscle. During slow injections very few changes in the ECG take place, although a slight decrease in heart rate is usually seen, even when the blood pressure is elevated. The reversal of blood pressure by adrenaline is not followed by a corresponding slowing of the heart rate, on the contrary, there is an increase. It seems that the action of adrenaline on the heart, as shown by the heart rate, is mediated in a different manner from its action on the rest of the circulatory system; possibly it reflects a difference between heart muscle and smooth muscle.

Summary.

In order to investigate the therapeutic possibilities in clinical conditions with increased sympathetic tone or adrenergic discharge experimental studies have been performed on the action of a new sympatholytic agent, dibenzyl- β -chloroethylamine. The drug is especially effective in eliciting a long-lasting blocking of adrenergic excitatory impulses when given by slow intravenous injection. Rapid injection even of moderate doses may however prove fatal. No toxic effects on brain, heart, liver or kidney has been observed with daily intramuscular injections given over several months. Despite possible unpleasant side-effects (tissue necrosis, cerebral irritation) it may be worth a clinical trial in selected cases.

This study has been aided by a grant from The Astra Company.

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Effect of Dibenzyl- β -Chloroethylamine on Metabolism in Rats.

By

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In the course of the experiments with dibenzyl- β -chloroethylamine (BIÖRCK 1947) it was recognized that there might be an inhibitory influence on metabolism if the drug in question had the sympatholytic properties described by NICKERSON and GOODMAN (1946). If there were a blocking of stimuli, which excite the sympathetic nervous system, it might be expected that the influence on metabolism of such stimuli would also be blocked. It is known that in animals deprived of their symphato-adrenal system the basal metabolism is reduced about 10 per cent. In a series of experiments which we have performed in order to investigate the eventual effect of dibenzyl- β -chloroethylamine on metabolism, we have obtained results, which indicate that intramuscular injection of the compound depresses the metabolism in rats. NICKERSON and GOODMAN (1946) report that dibenzyl- β -chloroethylamine is effective by all routes, and they state that excitatory adrenergic responses are blocked and reversed in mice, rats, rabbits, cats, dogs and humans. One of us has shown in an earlier paper that in cats and rabbits slow intravenous injection of the aqueous solution blocks the adrenergic influence on blood pressure whereas with intramuscular injection less effect is seen. We have not investigated these changes in rats. We have, however, found a decrease in the metabolic rate following intramuscular injection suggesting a degree of sympatho-adrenal block.

Method.

We have studied the oxygen consumption of rats and their RQ before and after injection of the drug. The method used was similar to the one described by BENEDICT (1909) with the modifications for the purpose of animal experimentation introduced by E. ABRAMSON. The principles of such an apparatus were recently described in this journal (CHRISTENSEN 1946).

We have used 5 adult rats and 5 immature rats. They all received a special diet which gives a value of the RQ of 0.859 and they had free access to food. The rats were weighed before each experiment. A series of control experiments were first performed, when the rats were injected with one ml. normal saline and after that a series in which they were injected with dibenzyl- β -chloroethylamine intramuscularly. 30 mg/kg bodyweight was given, diluted with saline to a total volume of one ml. The injections were given one hour before the experiment started. The length of the experiment was in all cases one hour. As far as possible the controls and the actual experiments were performed at the same time in relation to meals, though some irregularities in the results may be caused by the difficulty in achieving this.

Table I.

Animal	O ₂ consumed, ml.		CO ₂ given off, ml.		RQ		O ₂ -consump./gr weight	
	Control	Ex-perim.	Control	Ex-perim.	Control	Ex-perim.	Control	Ex-perim.
11665 A...	407	390	369	321	0.907	0.825	1.06	1.01
11768 B...	407	350	339	453	0.832	1.359	1.05	0.91
11534 D...	498	327	448	360	0.900	1.102	1.32	0.92
11810 C...	471	382	372	372	0.789	0.974	1.09	0.88
11746 C...	460	337	388	427	0.842	1.267	1.14	0.83
12764 B...	298	278	251	252	0.842	0.906	1.18	1.07
12617 B...	360	237	300	249	0.833	1.055	1.30	0.95
12753 A...	278	274	230	257	0.827	0.938	1.06	1.02
12728 E...	329	329	265	284	0.805	0.863	1.17	1.16
12760 E...	415	286	345	365	0.831	0.946	1.36	1.24

Results.

The table shows that the oxygen consumption diminished in relation to the controls when the rats were injected with dibenzyl- β -chloroethylamine. Only one animal showed the same value before and after injection. The CO₂-output increased in five animals, decreased in three and was unaltered in two animals. The RQ increased in nine animals and decreased in one. Calculated per gram bodyweight, the oxygen consumption decreased after the

injection in all animals. The high RQ values in some of the cases may be due to respiratory stimulation.

Discussion.

It appears from the above experiments, that injection of dibenzyl- β -chloroethylamine intramuscularly in rats is followed by a decrease in oxygen consumption in comparison with the oxygen consumption after injection of an equal volume of normal saline.

This might be due to a blocking effect on the excitatory adrenergic responses or to some effect on cerebral centers by the dibenzyl- β -chloroethylamine. It should, however, be born in mind that recent investigations by SELYE have led him to the conclusion, that the so-called "alarm reaction" in response to drugs, surgical interventions etc. causes a decrease of the metabolism during the "shock phase". Although there have been no indications that the injected animals in this series have felt any discomfort after the injections, this explanation cannot be altogether excluded.

Summary.

Intramuscular injections of dibenzyl- β -chloroethylamine in ten white rats caused a decrease of oxygen consumption during the second hour after the injection in comparison with the oxygen consumption after intramuscular injection of normal saline. The significance of the observation is discussed.

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Studies on Plasma-Prothrombin Following Pancreatectomy.

By

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When about ten years ago methods were found for the determination of the prothrombin content of the blood, interest was chiefly directed towards the lack of prothrombin in K-avitaminosis. It was, however, soon realized that affections of the liver alone might cause a deficiency in prothrombin without a co-existing lack of vitamin K. This discovery implied that the liver contributed towards the formation of the prothrombin, which has also been corroborated by experimental investigations. WARNER, BRINKHOUS and SMITH (1936) thus showed that, under the influence of chloroform, impairment of the liver parenchyma causes a fall in prothrombin. WARNER (1938) and WARREN and RHOADS (1939) also found a fall in prothrombin in experimental animals following partial and total hepatectomy, respectively.

The first report on the occurrence of K-avitaminotic deficiency of prothrombin in sprue appeared in 1939 (ENGEL; BUTT, SNELL and OESTERBERG as well as HULT). The cause was a defective absorption of the fat soluble vitamin K from the intestine. Later KOLLER, among others, has shown that K-avitaminosis may also occur in symptomatic steatorrhoea to the same extent as in the idiopathic steatorrhoeas. In affections of the pancreas lack of prothrombin may therefore be expected to occur as a consequence of a K-avitaminosis caused by pancreatogenic steatorrhoea. SPROUT and SANDERS (1942) furthermore have found a distinctly decreased content of vitamin K in the liver after pancreatectomy

Table 1.

Fasting blood-sugar after Crecelius-Seifert in mg %.

Days after operation	Dog nr 1	Dog nr 2	Dog nr 5	Dog nr 6
1	259	259	262	262
2	230	235	230	260
3	362	279	242	251
4	400	270	255	269
5	35	329	230	261
6	301	349	270	
7	221	271	397	
8	124	175	220	
9	69	263	253	
10	220	330	153	
11	151	182	28	
12	126	158	253	
13	81	30	191	
14	270	122	169	
15	211	100	—	
16	97	82	150	
17	—	—	180	
18	105	125	355	
19	315	308	296	
20	240	340	319	
21	229	400		
22	315	165		

in cats. BEGTRUP (1947) has recently published a case of prothrombin deficiency in a patient with an affection of the pancreas. Here, however, the deficiency of prothrombin was not affected by parenteral administration of vitamin K. Hence, no K-avitaminosis existed and the possibility had to be allowed for that in some way or other the pancreas is necessary for the formation of prothrombin in the organism. In order to examine this question it was decided to remove the pancreas experimentally on dogs and then follow the prothrombin-level in the blood.

For these experiments six dogs have been used. On four of them total pancreatectomy was performed. one was used as control without being operated on, and one was used as standard for the prothrombin determinations. The pancreatectomy has been performed under guidance of the classic experiments by MERING and MINKOWSKY. The operations have lasted about two hours, and the animals have been anesthetized with ether. Ether-anesthesia is stated to be without influence on the prothrombin level of the blood, provided that jaundice is not present (STEWART, ROURKE and ALLEN). Immediately after the operations 50,000 units of penicillin have been administered to the animals in order to prevent infections. The postoperative course has been without complications except in the case of dog Nr 4, which died

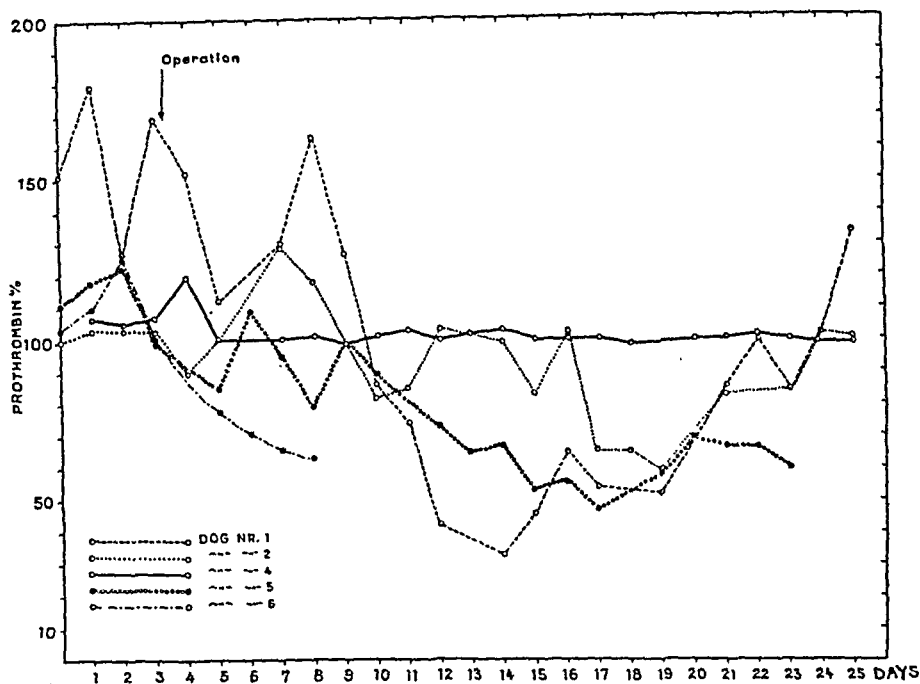


Figure 1.

Prothrombin percent after pancreatectomy in dogs. (Dog nr 4 as control without operation.)

on the sixth day after operation from an embolism in the left pulmonary artery.

During the whole of the experimental period the dogs have been fed on "black bread" (rye), "liver paste" and half a liter milk daily.

The blood-sugar has been determined with the method of Crecelius-Seifert. The determinations have been entered in Table 1. In the pancreatectomised dogs the fasting blood-sugar was somewhat high considering that the fasting blood-sugar of normal dogs is about 30 mg%. Insulin has been given postoperatively in the form of Insulin retard "Leo". The daily doses have been 20—50 International units. Postoperatively the urine of the dogs always on examination contained sugar, but never ketone-bodies.

The operated dogs have at no time presented jaundice neither has their urine been coloured by bile. At the termination of the experiments biopsy of the liver has been performed and it has been ascertained that the pancreas had been removed completely.

Blood for the prothrombin determinations has been taken by venipuncture, and the determinations have been made according to the method of THORDARSON (1941), THORDARSON, BEGTRUP and HANSEN (1943). The results are stated in Figure 1.

From figure 1 it appears, that the prothrombin values show an uncertain decrease immediately after the operation. About the

3rd—5th day an equally uncertain remission appears; but thereafter a fall in prothrombin is seen, the level coming down to definitely subnormal values. Towards the end of the experiments a rise is again observed in two of the surviving dogs. Even if at this point vitamin K is administered to the animals (an account of which will be given later), this increase seems to be spontaneous. Dog nr 6, which died on the sixth day after operation, showed an evenly progressive fall in the prothrombin values.

The fall in the prothrombin values after pancreatectomy is moderate. It moreover lasts at least a week before definitely subnormal values are seen. In cases of experimental partial or total removal of the liver the prothrombin-fall, which was very pronounced, occurred already in the course of 6—24 hours (WARNER, WARREN and RHOADS). If also the fact is taken in consideration, that prothrombin supplied by transfusion quickly disappears from the blood-stream, it appears that a formation of prothrombin must still take place in the animals after pancreatectomy. *The pancreas is therefore not essential to the formation of prothrombin in the organism.*

The experiments show, however, an uncontestable fall in the prothrombin values. In order to show, that this fall is not due to a K-avitaminosis caused by diminished absorption of vitamin K owing to steatorrhoea, a water soluble synthetic vitamin K-preparation has been administered to the dogs, partly perorally and partly parenterally. The preparation used was K-Vital "Leo" (Methyl-1,4 naphtoquinonebisulphite), in which 1 mg corresponds to about 20,000—25,000 Dam units. The doses and results are stated in table 2.

Table 2.

Prothombin percent in pancreatectomised dogs before and after administration of a water-soluble vitamin K preparation.

Dog nr	Prothrombin %	mg K-Vital	Prothrombin % 24 hours after
1	33	1	46
1	65	10	54
1	85	5	100
1	84	20	102
2	83	1	84
2	84	10	102
5	53	1	54
5	58	5	69
5	66	20	66

In the examined cases the results exclude that the low prothrombin-level is due to a K-avitaminosis. It can, however, not be excluded, that the large doses of K-Vital have influenced the course of the curves, but the reaction to the administration of the vitamin K-preparation are so little pronounced, that the effect definitely differs from that seen in cases of K-avitaminosis.

In the present experiments a vitamin K resistant lack of prothrombin has thus been found just as in the clinical case of pancreas-disease which occasioned the experiments. In the clinical case no trace of liver-disease had been ascertained. At the termination of the experiments microscopy of the liver from the dogs was performed by Dr. med. VIGGO ESKELUND. Staining with hematoxylin-eosin showed a total lack of inflammatory changes, and fat staining with sudan showed the following changes:

Dog Nr 1: Considerable diffuse fatty infiltration more pronounced peripherally than centrally.

Dog Nr 2: Slight fatty infiltration, yet distinctly more than normal.

Dog Nr 5: Rather pronounced diffuse fatty infiltration.

Dog Nr 6: Slight diffuse fatty infiltration.

In all the pancreatectomised dogs a more or less pronounced fatty infiltration was thus found. Hence it appears, that the liver has shown signs of steatosis in accordance with previous findings in pancreatectomised experimental animals (among others MONTGOMERY, ENTEMAN, CHAIKOFF; and NELSON, ENTEMAN, CHAIKOFF and MONTGOMERY). The liver has thus not been absolutely normal, and it is probable, that both *pancreatectomy and certain diseases of the pancreas in which the function of the gland ceases may influence the liver in such a way, that its prothrombin-forming capacity is more or less reduced.* That certain signs of impairments of the liver have not been found in patients with pancreas-disease does not mean that the liver's prothrombin-forming capacity has been intact.

BORGSTRÖM has assumed, that transmission of trypsin to the blood in acute pancreas-affections is the cause of low prothrombin values in these diseases. It is possible, that this explanation is correct in certain pancreas-affections; but the experiments with pancreatectomy on dogs just described show that lack of pancreas-function — whether due to lack of external or internal secretion — is able in other ways to compromise the formation of prothrombin, presumably by influencing the liver. This possibility

must therefore also be taken into consideration in clinical cases of prothrombin deficiency in pancreas-disease.

Conclusion.

1) The pancreas is not essential for the formation of prothrombin.

2) Pancreatectomy in dogs leads to a fall in the prothrombin values of the blood, presumably by compromising the prothrombin-forming capacity of the liver.

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Protein Metabolism of Tissue Cells in vitro.

6. The Accessory Growth Substances.

By

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In investigations carried out during the last years (cfr. FISCHER 1947), we have shown that two types of substances are essential to multiplication of tissue cells in vitro. The first type consists of the high-molecular substances present in embryonic tissue first demonstrated by CARREL (1913) and by us prepared as a nucleoprotein fraction and termed "embryonin" (FISCHER and ASTRUP 1943). The other type consists of low-molecular substances present in a variety of organic material, thus in blood serum, kidney tissue, yeast and barley malt (FISCHER 1941, FISCHER and ASTRUP 1942, ASTRUP, FISCHER and VOLKERT 1945), and which later could be partially purified (ASTRUP and FISCHER 1945, 1946).

When tissue cells are placed in dialysed culture media (containing glucose) they disintegrate in the course of 24 hours even in the presence of embryonin. In media containing the low molecular substances embryonin creates rapid growth of the tissue fragment (measured by an increase of the area). The low-molecular substances alone produce almost no growth, the cells mainly survive in good condition, responding immediately on the addition of embryonin with an increase in growth.

The low molecular substances evidently play a fundamental rôle in the maintenance of the tissue cells while embryonin only seems of importance to the multiplication of the cells and not

to the maintenance of the life of the cells. Embryonin therefore may act as a catalysing principle for the growth of the tissue, while the low-molecular substances serve as a basic nutriment for the cells. For this reason we call embryonin a *growth promoting substance* while the low-molecular components are regarded as *accessory growth substances*, and we use these terms in order to distinguish between the modes of action of the two different groups of substances on the tissue cells.

In a recent paper we have described the removal of the fermentable sugars from barley malt extracts by means of a treatment with yeast (ASTRUP, FISCHER and ØHLENSCHLÄGER 1947). With these preparations we were able to study the importance of different sugars for the growth of tissue cells, and to obtain a purified active product as a dry powder. It is thus possible to remove considerable quantities of inactive material without interfering to any great extent with the growth properties of the preparations. It is the purpose of the present paper to make a further contribution to the knowledge of these accessory growth substances.

1.

Before entering upon the experimental details it may be of value to consider some questions concerning "*accessory growth substances*", which term is a purely arbitrary one. Thus it must be pointed out, that many more substances may be "*accessory growth substances*" for a culture of tissue cells than for a whole organism, as the cells may be unable to produce all of the intermediary metabolites present in the fluids of the whole organism originating from enzymatic processes in special organs, *e. g.* in the liver. If we take into account all low-molecular ions and neutral substances of the *intercellular milieu* possessing a definite influence upon the cellular metabolism, the term "*accessory growth substances*" may embrace alle these types of low-molecular substances for which the cell membranes are permeable. For any tissue in question it is therefore of importance to disclose, which of the said substances acting upon its metabolism, it can be without and which substances it is unable to produce as intermediates in its own metabolic processes. The last mentioned substances must be added to the culture medium for the tissue in question, and must therefore be termed "*accessory growth substances*" in a restricted sense of the term.

The "activity" of an extract is therefore an expression for the joint action of its different components on the tissue cells. Some of these components may be "accessory growth substances" in the restricted sense; others may be removed from the solutions without interfering with the growth of the tissue fragment, as the cells themselves may be able to produce these intermediates.

Still other components may be impurities of no significance whatever to the cells, and others may be inhibitors. Further, the concentration of the components may be of importance, as some substances, active components or impurities, may act as inhibitors at increased concentrations. This should be taken into consideration when evaluating the experimental results.

The components acting upon the cells may be divided into the following groups:

A. Inorganic salts:

1. Na^+ , K^+ , Ca^{++} , Cl^- , HCO_3^- , HPO_4^{--} and similar ions influencing cellular permeability, pH and colloidal state of the proteins.
2. Mg^{++} , Mn^{++} , Cu^{++} , Zn^{++} , Fe^{++} and similar ions, with special functions in cellular metabolism, mainly as components of prosthetic groups of enzymes.

B. Organic substrates:

1. Low-molecular carbohydrates, mainly glucose.
2. Low-molecular peptides and free amino acids.

C. Metabolites:

1. Intermediate breakdown products from the carbohydrate series.
2. Intermediate products from the oxidation of fats.
3. Intermediates from the oxidative de-amination of amino acids, as aldehydes, ketones and keto acids free or in the form of enol-phosphates.
4. Phosphate transmitters, adenosine triphosphate and systems of similar kinds.
5. Redox systems, acids of the Krebs cycle, cozymase, thiamine, flavines and others.

D. Other specific biokatalysts acting in a very high dilution, like pantothenic acid and biotin in yeast systems.

E. Inhibitors.

This list is not complete, as the intermediates from other lesser known systems, as *e. g.* the amino acid metabolism and transmethylation, may be included, as well as still unknown processes of importance to the metabolism and growth of isolated tissues.

The tissues are cultivated in a mixture of dialysed plasma and dialysed embryonic extract. Dialysed blood serum is added as fluid phase. All the high-molecular components are therefore retained in the media, including the growth promoting substance embryonin. Further, the dialysed proteins in the medium may serve as a source for the

amino acids necessary for the metabolism of the cells, provided the isolated cells are able to make this source available, a question which has previously been discussed in detail, ASTRUP, FISCHER and VOLKERT (1945).

The presence of Tyrode-solution in the culture medium provides the system with some inorganic ions of group A and with glucose. The removal of these substances from the active preparations may therefore be regarded as the first step of purification; thus the removal of fermentable sugars present in the extracts of barley malt has been accomplished recently, ASTRUP, FISCHER and ØHLENSCHLÄGER (1947). In this manner it was possible to study the ability of different sugars to act as "accessory growth substances", and it was found that only glucose, mannose and fructose showed any considerable activity.

Substances from the other groups act as growth substances under the experimental conditions chosen if the tissue cells are unable to produce them through their own metabolic processes, or otherwise to make them available. Thus it may be possible, that not all these accessory components are present in the normal media in a free state, but *e. g.* combined with proteins. If the compound formed is a relatively non-dissociable compound, then the substance in question will not be removed by the dialysis.

In this manner some of the substances reckoned to be of low-molecular character may be retained in the dialysed media, and consequently be available for the cells for growth purposes. This means that the addition of these substances to the media is not necessary for obtaining normal metabolism and growth of the tissues.

That this may be true is indicated by our purification experiments, in which we could show that a considerable purification could be accomplished without interfering to any large extent with the action on the cells. This result was unexpected. For example, we assumed that the different vitamins which are known to be necessary for a whole organism also are essential to a tissue fragment under our experimental conditions, while so far we have been unable to discover any influence of any of the vitamins tried (B-group and vitamin C) on the growth. Only two explanations remain: either are the vitamins present in the dialysed media as non-dissociable compounds with high-molecular substances, or the culture period is too short to disclose any deficiency. Another point must also be taken into consideration here, viz. that the number and amount of vitamins essential to an isolated tissue fragment may be different from that essential to a whole organism, where special organs or specialized tissues, on which the life of the organism is dependent, may use vitamins which are not essential to other, less specialized tissues, or use them in lesser amounts. Another explanation — rather improbable as it would seem — remains, namely that the dedifferentiation of the tissues during the cultivation *in vitro* makes the cells capable of surviving and multiplying on media of a simpler composition.

One must be cautious in judging the course of fractionation of a crude preparation from the growth of tissue cells *in vitro*. It must

be emphasized that tissue cultures are very unfavourable objects for a quantitative test of the activity, allowing only a very rough estimation of the potency of the different fractions. It is only possible to say something definite regarding the activity when it is approaching zero, in which case the product may be discarded, and this is only possible after testing different concentrations of the preparation in question, as there seems to exist a threshold concentration below which a fraction is practically inactive, while it may be rather potent at higher concentration. For this estimation both the growth rate (as compared with that of a sister culture as control) and the morphological appearance must be taken into consideration. It is evident, that with this test method the purification may proceed only at a slow rate. It is necessary to test every new fraction and fractionation method over and over in order to obtain reliable results. The technical difficulties met with in tissue cultivation further limit the number of single experiments to which a preparation may be subjected simultaneously. For comparison it may be mentioned that in the purification of pantothenic acid it was possible accurately to determine the potency of the fractions within a concentration range of one to several thousands using a simple yeast test (WILLIAMS, TRUESDAIL, WEINSTOCK, ROHRMANN, LYMAN and Mc.BURNEY (1938)).

For this reason we have on several occasions during our work considered the possibility of designing a new test method. We have thus made orientating experiments with measurements of the metabolism of tissue slices using the Warburg technic, and we have also tried to measure the proteolytic activity of isolated tissue in the presence or absence of our substances. All without much success. It must also be pointed out that, notwithstanding the ability of a new test method to function in the presence of our more or less purified preparations, a new difficulty is introduced, as the new test may depend on the presence of other substances, than those acting on the animal tissue cells. In this case a new problem arises during a purification procedure, namely that of the identity or non-identity of the active fractions as measured by the different test methods. A further uncertainty is to be found in the circumstance that we still do not know in which processes of the metabolic activities of the cells the substances in question participate.

Further, we do not know if the response of the tissue cultures on the addition of an active preparation is due to the presence of one, a few or a considerable number of chemical substances acting in conjunction to restore the growth ability of the tissue fragments.

2.

Experimental.

1. Barley Malt Concentrate.

In our last paper (ASTRUP, FISCHER and ØHLENSCHLÄGER 1947), we described experiments with malt extracts fermented

with yeast in order to remove the carbohydrates present. In order to prepare larger quantities a concentrate was made in the following manner in a commercial plant:

2 kg barley malt powder was stirred mechanically $1\frac{1}{2}$ hours at room temperature with 5 l of water. Then 15 l of 96 % ethyl alcohol was added. After standing until the next day it was filtered, and concentrated in vacuo on waterbath ($40-50^{\circ}$) to a volume of about 1 l. After decantation from the lipids separated it was concentrated in the same manner to 250 ml. 100 kg barley malt was worked up in this manner.

2. A Dry Preparation.

The treatment of this concentrate with yeast (after dilution to the original volume) now yielded practically inactive solutions. We tried in vain to disclose the cause of this failure. The concentrate as furnished to us contained less carbohydrate and nitrogen than usual ($V-396,0$: 10.7 mg sugar and 0.47 mg N per ml). We tried to reduce the amount of yeast used for fermentation, but without much success. The amount of non-fermentable reducing substances was about the same as usual ($V-393,5$: 4.75 mg sugar per ml). We therefore abandoned the fermentation as a method for the purification of the active substances, and tried a direct precipitation. This was successful, probably because the carbohydrate content of the crude extracts was lower than that of the extracts used before. The dry preparation obtained in this manner was more colourless than before, and dissolved in water to a clear, yellow solution without leaving insoluble substances behind. The following procedure was followed:

To 500 ml of a concentrate was added 50 ml 2—n NaOH (alkaline on phenolphthalein) and the solution precipitated with 3,000 ml 96 % ethyl alcohol. After standing for a few hours the supernatant was discarded by decantation, and 500 ml glacial acetic acid added to the viscous precipitate. After standing over-night the mixture was cautiously heated in a water bath until about 60° with intermittent shaking. Most of the precipitate was dissolved by this treatment. After slowly cooling to room temperature and standing until the next day the supernatant (520 ml) was poured from the sticky precipitate and precipitated with 5 vol. (2,600 ml) of abs. alcohol with mechanical stirring. After a few hours the mixture was filtered on a Büchner funnel, treated with abs. alcohol and dry ether and dried in vacuo. Yield 13.0 g, corresponding to 1.3 mg substance per ml of the original solution (10,000 ml). Nitrogen content: 4.25 % ($V-403,2$).

The yield of dry substance depends on the sugar content of the original concentrate and varies between 1.2 mg to 4.9 mg substance per ml original solution. The nitrogen content lies around 2—4 % and the sugar content at about 30—40 %. Phosphorus content about 1 %. Cl is absent. The preparation contains most of the activity of the original material, see Fig. 1.

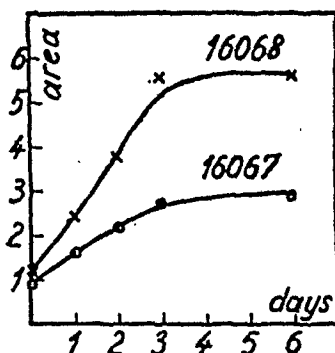


Fig. 1. Action of a dry preparation from malt: V—406, 2, 4.33 mg per ml original solution. 216 mg dissolved in 50 ml Ringer's solution, neutralized and sterile-filtered. Culture No. 16067: No addition. Culture No. 16068: Addition of 0.2 ml solution.

3. Barium Salts.

The dry preparation could be further purified by fractionation as barium salts with ethanol. In this manner a middle fraction containing most of the activity could be isolated, but it was difficult to prepare the remaining fractions completely inactive.

V—401, 21: 9.4 g V—401, 2 (a dry preparation corresponding to 2,000 ml original solution) was dissolved in 25 ml H_2O and precipitated with 2.5 g barium acetate dissolved in 10 ml of water. After centrifugation it was washed once with 5 ml water and dried with alcohol and ether. Yield 1.11 g containing 1.35 % N and 4.23 % P. Shows no activity (after removal of Ba^{++} with sulphate).

V—401, 22: The centrifugate and the wash water from V—401, 21 were precipitated with 1 vol. abs. ethanol (40 ml) and dried with ethanol and ether. Yield 0.61 g, 1.86 % N and 3.28 % P. Inactive.

V—401, 23: To the centrifugate from V—401, 22 there was added 2 vol. of abs. ethanol (80 ml). Yield of precipitate 1.04 g containing 4.25 % N and 0.597 % P. Tried in 4 times the original concentration it showed considerable activity, Fig. 2.

V—401, 24: The mother liquor was precipitated with an additional 2 vol. of abs. ethanol (total 5 vol.), 1.72 g precipitate. 2.82 % N and 0.452 % P. Not very active.

V—401, 25: The mother liquor was precipitated with 2 vol. dry ether. 3.54 g precipitate. 0.627 % N and 0.458 % P. Almost inactive.

From these experiments it is seen that large amounts of inactive substances containing phosphorus were removed in the most insoluble fractions. The activity followed the nitrogen-rich fractions of medium solubility. The most soluble fractions were inactive and contained only small amounts of nitrogen. When using larger amounts of material it was difficult to prepare the

less soluble fractions in completely inactive form; best results were obtained by the following procedure.

V — 4 0 7, 3 1 : 20 g V — 4 0 6, 2 (a dry preparation corresponding to 10,000 ml original solution) was dissolved in 100 ml H_2O and 10 g barium acetate dissolved in 50 ml H_2O was added. After centrifugation the precipitate was dispersed in water, made alkaline to phenolphthalein with 2 — n NaOH and Ba^{++} removed by a surplus of 5 % sodium carbonate. After centrifugation and filtration the mixture was diluted to 250 ml ($40 \times$ the original concentration). Contained 0.578 mg N/ml

corresponding to $14.5 \mu g$ N per ml original solution. Showed practically no activity.

V — 4 0 7, 3 2 : The centrifugate from V — 4 0 7, 3 1 was precipitated with 6 vol. of abs. alcohol (900 ml). After decantation the sticky precipitate was dissolved in water, some insoluble substance removed by filtration and diluted to 200 ml ($50 \times$ concentration). Contained 2.023 mg N/ml corresponding to $40.5 \mu g$ N per ml original solution. Considerably active, see Fig. 2.

V — 4 0 7, 3 3 : The mother liquor was evaporated in vacuo and diluted to 200 ml ($50 \times$ concentration). It contained

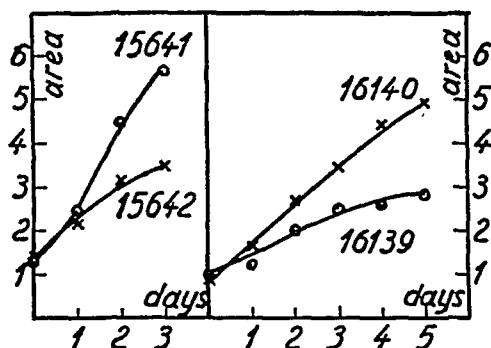


Fig. 2. a) Action of a medium soluble barium salt from malt, V — 4 0 1, 23 (culture No. 15641) compared with the action of a more soluble Ba-salt V — 4 0 1, 25 (culture No. 15642). b) Culture No. 16140: Action of an alcohol-precipitated barium salt V — 4 0 7, 3 2. Culture No. 16139: No addition.

tained 1.038 mg N/ml corresponding to $20.8 \mu g$ N per ml original solution and was inactive.

4. Further Properties of Malt Extracts.

Treating the dry active barium salts with methanol several times removed about half the amount as soluble inactive substances, containing only minor amounts of nitrogen (V — 4 0 5, 3 2 : 0.79 % N). The insoluble residue was rich in nitrogen and showed considerable activity (V — 4 0 5, 3 1 : 5.42 % N).

Treatment of a dry preparation with colloidal ferric hydroxyde removed a little sugar and some nitrogen without influencing the biological activity (V — 3 9 8, 2 : 1.93 mg sugar and 0.160 mg N per ml, Fe_2O_3 -treated (V — 3 9 8, 2 — F): 1.20 mg sugar and 0.096 mg N per ml).

Treatment with methanol acidulated with HCl dissolved most of the dry preparation. The solution yielded on addition of dry

ether a precipitate possessing considerable activity, but addition of a solution of sodium in methanol until alkaline reaction precipitated most of the active components in a more purified state (V—399, 2: 1.22 mg sugar and 0.117 mg N per ml. Sodium salts insoluble in methanol (V—399, 53): 0.28 mg sugar and 0.064 mg N per ml).

The sodium salts insoluble in methanol (V—403, 31) contained 5.67 % N, of which 1.30 % was amino-nitrogen before hydrolysis by 1-n HCl, and 1.99 % amino-nitrogen after hydrolysis for $\frac{1}{2}$ hour at 100°.

By means of a zinc precipitation it was possible to purify the barium salt further and to increase the nitrogen content considerably:

V—404, 231: 2.0 g barium salt (V—404, 23) was dissolved in 5 ml H_2O , and precipitated with concentrated Na_2SO_4 -solution. The $BaSO_4$ removed by centrifugation was washed with 1 ml H_2O and to the combined solutions 1 g zinc acetate dissolved in 2 ml H_2O was added. The mixture was precipitated with 50 ml CH_3OH and the precipitate isolated by centrifugation and treatment with abs. alcohol and dry ether. Yield 0.87 g. The precipitate was active and contained 8.80 % N. The mother liquor was also considerably active, however, and yielded on addition of abs. alcohol (1 vol.) 0.49 g dry substance containing 1.09 % N (V—404, 232). By converting the zinc salt again to a barium salt (by means of $Ba(SH)_2$ -solution) a barium salt, containing 11.05 % N but being less active than the original zinc salt, could be obtained. (V—404, 235.)

Treatment with 90 per cent phenol of the barium salts dissolved in water yielded a nitrogen-rich phenole fraction (V—406, 423: 0.045 mg N/ml original solution) and a nitrogen-poor aqueous layer (V—406, 422: 0.016 mg N/ml). None of the solutions showed any considerable activity, but united they seemed rather active. This indicates the possible presence of two kinds of substances essential for the biological activity of the extract. (V—405, 541, V—408, 31.)

Acetylation with acetic anhydride and sodium hydroxyde at 0° in aqueous solution did not decrease the activity (V—333, 12 V—405, 51).

5. Accessory Growth Substances from Embryonic Calf Tissue.

The low molecular accessory growth substances must be assumed to be present abundantly in the embryo tissue, and we therefore tried this as a source that might yield the substances

in a purer condition than the malt extracts and possibly also of a kind more natural to the animal tissues investigated. We made a dialysate from minced calf embryo and obtained immediately a product of considerable activity, and with the morphologic appearance of the cells superior to the most active malt extracts previously tried. We tried various other means of obtaining protein-free solutions, but they all yielded products inferior to those obtained by dialysis. This was true, for example, of methods involving trichloroacetic acid, metaphosphoric acid and acid alcohol. This failure of the usual methods for the removal of proteins was probably due to the nature of the proteins of the embryo extract which contained large amounts of gelatine.

Metaphosphoric acid was hydrolysed by heating at slightly acid reaction and removed as barium phosphate at alkaline reaction. Trichloroacetic acid was removed by concentration in vacuo, solution in glacial acetic acid and methanol and precipitation of the active product by means as abs. alcohol. Although products of a considerable activity were obtained (V — 509, 1; V — 524, 1) the properties of the substances were inferior to those obtained by dialysis, it was especially the contents of gelatine which made the treatment of the substances difficult. It was therefore decided to use only dialysates for the further investigations.

V — 501, 1: Muscles from calf embryos were passed through a meat chopper and extracted for one hour with one volume of Ringer's solution. After passing through gauze 5.5 l extract was obtained corresponding to 2.75 l embryo pulp. The extract was dialysed in cellophane tubings against an equal volume of distilled water at 0° for three days with addition of toluene. The dialysate, corresponding in concentration to 1.37 l embryo pulp, was evaporated in vacuo to dryness on a water bath (50°) and the residue treated with methanol-acetic acid (1:1) 80 ml. The filtrate was precipitated with 5 vol. of abs. alcohol, filtered on a glass filter and treated with abs. alcohol and dry ether. After drying in vacuo over H_2SO_4 and NaOH 18.6 g of a white powder resulted, containing 2.95 % N, 2.60 % total P and 1.20 % inorganic P. 415 mg was dissolved in 25 ml physiol. NaCl, neutralised and steril-filtered.

It was found very active on the tissue cultures yielding almost normal growth and appearance, see Fig. 3. Dilution of such a preparation 1:10 did not completely reduce its complementary action on dialysed media.

An extract of barely malt used in 10 times the usual concentra-

tion did not produce increased growth effect and was still inferior to the embryo preparation. Addition of a concentrated malt extract to the embryo preparation did not show any sign of the presence of inhibitory agents in the malt extract, Fig. 4.

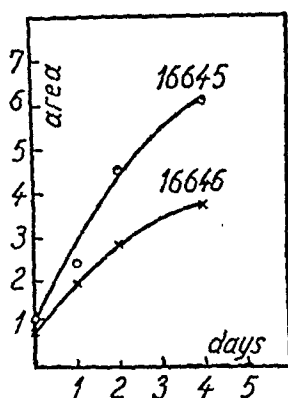


Fig. 3. Action of a dry preparation (V—501, 1) from calf embryo dialysate undiluted (culture No. 16645) and 10 × diluted (culture No. 16646).

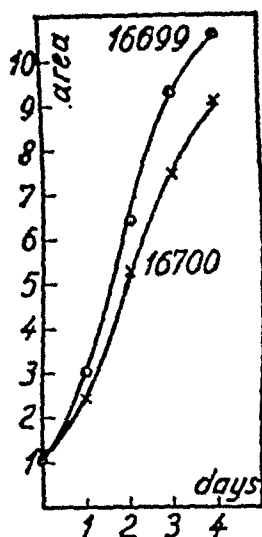


Fig. 4. Action of V—501, 1 without (culture No. 16699) and with (culture No. 16700) addition of an extract from barley malt (V—425, 1).

The embryos used may be of different size and may be kept at 0° over night and still yield excellent products. The dialysis may be extended over two to five days and the water changed. The cellophane tubings may be used several times when rinsed and kept in toluene-water. In order to obtain active preparations it is important to add toluene or xylene to the embryo extract and treat the extract thoroughly with these additions before placing in the cellophane casings. When no toluene was added quite inactive preparations were obtained, probably due to bacterial decomposition of the active components; no toxic products were present (V—507). The embryo extract could be stored with toluene a few days at 0° without loss of activity (V—511, 3).

The active substances present in the products obtained in this manner possess considerable stability. Treatment with 0.1 — n HCl or NaOH for half an hour at 100° did not result in any significant decrease of activity. The same was found when treating with 1.0 — n HCl, but 1.0 — n NaOH produced almost inactive

solutions (V — 5 0 0). The yield of dry substance was as a rule about 10 mg per ml embryo pulp, with due corrections for loss by extraction and dialysis. Beef muscle also yielded active products (V — 5 0 8). Also ultrafiltrates of the embryo extract were active (V — 5 1 1), method of BRANDT REHBERG (1943). Chicken embryos also yielded active dialysates, V — 5 1 5. By electrodialysis of the dry preparation about $\frac{1}{3}$ was isolated as acid components, carrying most of the activity, V — 5 1 7.

The properties of the embryo preparations were as follows:

Normal growth and appearance of the tissue cultures was obtained, when adding amounts corresponding to the concentrations in the embryo tissue. At lower concentrations (1 : 10) the activity decreased. The activity was not destroyed by keeping the embryos or the pulp at 0° for a few days. Autoclaving at neutral reaction, treatment with 0.1 — n acid or 0.1 — n alkali at 100° resulted only in minor losses in activity. The same was the case when treating with 1.0 — n HCl, while treatment with 1.0 — n NaOH resulted in inactivation. The dry preparation contained large amounts of phosphorus. Thus V — 5 0 1, 1 contained 2.60 % total P, of which 1.20 % was inorganic P; 0.07 % 7 min. P. and 0.40 % 20 min. P. Treatment with Ba-acetate precipitated all the inorganic P, but only small amounts of N. The remaining solution retained most of the nitrogen and the activity. By treatment with glacial acetic acid all the activity was transferred to the solution. By electrodialysis an active acid fraction could be obtained.

Some of the properties of our active preparations indicate a similarity with the substances involved in the overcoming of adult tissue dormancy studied by SIMMS and STILLMAN (1937) but it is too early to speculate about their identity.

Summary.

1. In a treatment of the problems concerning the growth substances acting upon animal tissue cells in vitro it is pointed out that it is necessary to distinguish between the nature and mode of action of the *growth promoting substances*, termed embryonin, which are high molecular protein fractions present in an embryo extract, and the *accessory growth substances* of low-molecular nature present in a variety of biological systems. Both of these

groups were found to be essential to the growth of tissue cells in vitro.

2. When studying the properties of the low-molecular substances several difficulties are encountered which are inherent in the test method available and therefore unavoidable. The meaning of the term "accessory growth substances" for tissue cultures in vitro is discussed.

3. The purification of the barley malt extracts previously investigated was carried further with only partial success. The activity of the fractions diminish as the purification proceeds. In some cases completely inactive fractions result, which when mixed regain a considerable activity indicating the presence of at least two groups of accessory growth substances essential to the tissue cells.

4. From embryonic extracts it is possible to isolate as a stable dry powder purified preparation which is superior to the best malt extracts in producing normal growth and appearance of the tissue cultures.

This work was aided by grants from "*Rask-Ørsted's Fond*", "*Teknisk Kemisk Fond*" and "*Ingeniørvidenskabeligt Fond*". The barley malt was placed at our disposal by the *Carlsberg Breweries*, and *A/S "Ferro-san"*, Copenhagen, aided us in the preparation of crude extracts.

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From the Physiological Institute, University of Lund.

The Effect of the Food Intake on the Morning Value of the Citric Acid Concentration of the Blood.

By

CURT WALLMAN-CARLSSON.

Received 31 May 1947.

This report covers one of several investigations of the occurrence and significance of citric acid in the human organism, which have been carried on for some time in different institutions in Lund. It is most closely connected to a paper by AGRELL (1946) concerning the blood citrate level as dependent on muscular work. The Thunberg method, which was also used in this investigation, is described by MÅRTENSSON (1940) and HAGELSTAM (1944). — The investigations on which this report is based were carried out in April—June 1946.

A relatively small part of the greater problem of how the citric acid content of the blood is affected by food on the whole was studied: the effect of food consumed in the morning before rising on the citric acid content. The aim of the investigations is primarily to elucidate some of the conditions necessary for the clinical use of the citric acid determination and the necessary precautions, especially when diagnosing certain diseases of the liver. The investigation has been confined within these limits.

The following information may be given concerning the persons examined. There were 4 males and 6 females ranging in age from a 9-year old school-boy to a 39-year old woman. All were in good health and consisted mainly of research workers or technicians.

The blood samples were obtained by puncturing the cubital veins and the citric acid analyses were performed according to THUNBERG.

The examinations were scheduled as follows:

The subjects were not allowed to eat anything after 7 p. m. on the day preceding the test. They spent the night at the laboratory in order to avoid a possible change in the Ci-values due to muscular efforts. Blood sample No. 1 was taken as soon as possible after awakening in the morning. The pulse was counted before the blood-test in order to get a rough idea of nervousness of the subject before the test. After the first blood sample was taken the subject was served a light breakfast in bed, consisting of one egg, 2 pieces of bread and butter, one with cheese and the other with corned beef, together with $\frac{1}{2}$ litre of milk. All subjects consumed the entire meal except No. 2 who did not eat the egg and Nos. 6 and 10 who left half of the milk. — According to an approximate calculation, the caloric intake was about 670 cal. (2 pieces of bread weighing 25 gr. 120 cal., 15 gr. cheese 40 cal., 5 gr. corned beef 8 cal., 10 gr. butter 76 cal., 1 egg 80 cal., and $\frac{1}{2}$ litre milk 350 cal., altogether 670 cal.) After breakfast the subject was allowed to remain in bed to rest for one hour, following which the pulse was counted again and blood sample No. 2 was taken.

The values are given in the Table below.

Table 1.

In Table 1 A gives the sex and age of the subjects, B the Ci-value before the meal, C the Ci-value after the meal, D the percentual change in the Ci-value, E the pulse rate on awakening, F the pulse rate 1 hour after the meal, and G the percentual difference in the pulse rate.

No	A	B	C	D	E	F	G
	Age						
1.	♂ 24	30	35	+ 17 %	64	68	+ 6 %
2.	♀ 19	30	35	+ 17 %	72	78	+ 8 %
3.	♀ 19	23	30	+ 30 %	68	72	+ 6 %
4.	♂ 9	40	42	+ 5 %	64	76	+ 19 %
5.	♂ 35	26	34	+ 31 %	60	62	+ 3 %
6.	♀ 39	25	33	+ 32 %	72	74	+ 3 %
7.	♀ 35	24	29	+ 21 %	60	60	0.0 %
8.	♀ 16	27	32	+ 19 %	68	84	+ 24 %
9.	♀ 21	24	28	+ 17 %	68	74	+ 9 %
10.	♂ 9	35	41	+ 17 %	70	76	+ 9 %

By means of comparing the values in column B and column C the effect of food on the Ci-value of the serum is obtained. The values in these two columns have been placed in relation to each other in column D, where the differences between B- and C-values are stated per cent.

All the differences are positive. Thus the result of the determination is that the intake of food seems to induce a clear increase in Ci-value of serum. The lowest increase observed is 5 %,

the highest 32 %. The mean value of the percentual increase is 20.6 %. — An increase is seen also in the case with an original value of 40. Here the increase was 5 %, which figure, however, lies within the limits of error and must be judged accordingly. The subject in question, the 9-year old boy mentioned before, was tested again (test No. 10), resulting in an increase of 17 % of the initial value of 35. — A special study of the Ci-value in children is, of course, indicated, as it is very probably generally higher than in adults. However, the young boy tested here showed on both occasions an increased Ci-value of serum after eating.

The pulse rate which was determined before taking of the blood samples showed an average increase of 9 %.

In order to ascertain that the increased Ci-value was caused by the food and not only by the fact that the subject was awake, control experiments were made without food on 5 different subjects, 4 of whom had belonged to the group originally examined. The other was a man aged 28.

The result of this control series appears in Table 2.

Table 2.

Column A gives the sex and age of the subjects, B the Ci-value on awakening, C the Ci-value after 1 hour, and D the percentual change of the Ci-value.

No	A	B	C	D
	Age			
1.	♀ 19	30	28	—7 %
2.	♀ 16	26	24	—8 %
3.	♂ 28	23	23	0.0 %
4.	♀ 35	26	26	0.0 %
5.	♀ 21	24	25	+ 4 %

The values of this control series show thus that the fact of being awake cannot alone cause the increase in the Ci-value of the blood which is observed to occur after the consumption of food.

Why does the intake of food produce an increase of the Ci-value of the blood? The resorption of the Ci contained in the food may be more or less important. The food used in our experiment contained a considerable amount of Ci, mainly due to the milk. Milk has a Ci-value of about 3 gr. per litre and the half-litre of milk which was part of the meal, contained thus more than 1 gr. of Ci. By the meal given the body is thus supplied with an

amount of Ci that is considerably greater than that of the blood, calculated to about 1 mg. per kilo body weight, *i. e.* 70 mg. The additional Ci-amount is thus more than 10 times that of blood serum. It is evident that the experiments should be made also with food as free from Ci as possible.

Our results do not exclude the possibility of the increase in the Ci-value of the blood being caused by a general increase in the metabolism. It is therefore desirable that the experiments are continued with food that is almost entirely free from Ci. It is also important that the blood sample for diagnostical Ci-analysis should be taken before any food is consumed. This is in fact merely a continuation of the method in practice.

SJÖSTRÖM, who introduced Ci-determination as a means of liver diagnosis, stresses, too, that the tests should be made under similar conditions, preferably in the morning on an empty stomach. This rule is none the less motivated by the fact that SJÖSTRÖM is uncertain whether food increases the Ci-values or not. .

LENNÉR has found in his obstetrical cases no noticeable influence of food in the Ci-value of the blood.

LEHMANN considers that blood samples for determining the Ci-value should be taken before rising in the morning and on an empty stomach. He says the following:

"The sample should be taken on an empty stomach because the food may contain Ci (vegetables *a. o.*). I think, however, that our view of the reason for the increased Ci in non-fasting patients must be revised. We have thus found that quite harmless meals, containing only a minimum of Ci, have caused increases in Ci amounting to values comparable to those observed in the most severe hepatitis". — According to LEHMANN this means that the increased Ci is not caused by the occurrence of Ci in the food, but rather by an increased intestinal activity and metabolism, respectively, in connexion with the consumption of food.

Summary.

The intake of food in the morning immediately after awakening causes an increase in the Ci-value of the blood serum, measured by Thunberg's enzymatic method. In a series of 10 subjects the lowest increase was 5 %, the highest 32 %, and the mean value 20.6 %. When the method is used for diagnostic purposes in liver diseases, blood samples should be taken on an empty stomach and under so similar conditions as possible.

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The Effect of Adrenaline and Related Compounds on the Permeability of Isolated Frog Skin to Ions.

By

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During recent years, this laboratory has taken up an investigation of the transfer of ions across the skin of amphibians and the regulation mechanism involved. It was found that the transfer of sodium and chloride through the skin of the axolotl was affected by injection of the neurohypophyseal principles (JØRGENSEN, LEVI and USSING, 1947). Moreover, the mere pricking of the skin with the injection needle induced a loss of salt from the animal. It is natural to correlate this phenomenon with the observation that the potential across frog skin has been observed to decrease as a result of stimulation of the nerves in nerve-skin preparations (see GILDEMEISTER, 1928). GREVEN (1941) showed that even psychic excitation, for example released by noise, may produce a decrease in skin potential of the intact frog. Furthermore SAITO (1931) observed that stimulation of the nerves and addition of *i. a.* adrenaline to frog skin increased its polarization capacity. These observations make it most probable that the skin permeability of amphibians may be influenced by sympathin and adrenaline. It was therefore attempted to study the effect of adrenaline on the ion permeability of the isolated frog skin by means of direct methods.¹

¹ Still unpublished experiments of Dr. H. H. USSING show that the potential across isolated frog skin decreases on addition of adrenaline to the Ringer solution bathing the inner side of the skin. I thank Dr. USSING for his kind permission to cite his results.

Material and Technique.

The experiments were performed on isolated skin from the thigh of the frog (*Rana temporaria*). The skin was turned inside out, the distal open end was closed by ligation, and the proximal end mounted on a celluloid tube c. 12 mm in diameter. At the start of the experiments the pouches thus obtained were filled with c. 1.5—2 ml water or dilute salt solution and placed in small vessels containing 10 ml aerated frog Ringer. Care was taken that the liquid surfaces were at the same level on both sides of the skin. The air stream used for aeration entered beneath the skin pouch and by hitting the skin pouch before reaching the liquid surface it maintained satisfactory stirring of the content. The Ringer solution did not contain bicarbonate, but it was buffered at pH c. 7.4 with sodium phosphate (c. 3 mM/l); in addition the Ringer contained 60 mg% of glucose.

The passage of salts through the skin was followed either 1) by chloride determinations or 2) by conductivity measurements on the content of the pouches. Chloride was determined according to REHBERG in the modification of SCHNOHR (1934) on $\frac{1}{2}$ ml samples. The conductivity was measured by means of a Philips Philoscop (a.c. 1000 cycles). In the following the resistance measured is expressed directly in concentrations *i. e.* in mM/l of a solution of NaCl which — at the same temperature (c. 22° C) — would have the same conductivity.

Results.

In the experiments to be described below the net movement of salt always went in the direction from the higher concentration in the Ringer solution to the lower concentration inside the skin pouches. Thus a net uptake of salt from the fluid bathing the physiological outer side of the skin was never observed. Usually the newly mounted skins showed a rather high permeability decreasing when the liquid in the pouches was exchanged (table 1).

Table 1.

Permeability of frog skin in $\mu\text{M Cl/h/cm}^2$.

	1	2	3
Freshly prepared skin pouches .	0.06	0.09	0.23
After renewal of pouches' content,	0.02	0.03	0.06

This seems to indicate that during preparation of the skin a substance is liberated which renders the skin more permeable to salts. After the content of the pouches was renewed once or a few times, however, the skin permeability was found to remain constant under

constant conditions for more than two hours (fig. 1). KROGH (1937) found that specimens of *R. esculenta* when kept in distilled water loose salts through the skin at a rate of c. $0.01 \mu\text{M Cl/h/cm}^2$. Comparing this figure with the result of the present measurement it is seen that the permeability of the isolated skin of *R. temporaria* is at least several times higher.

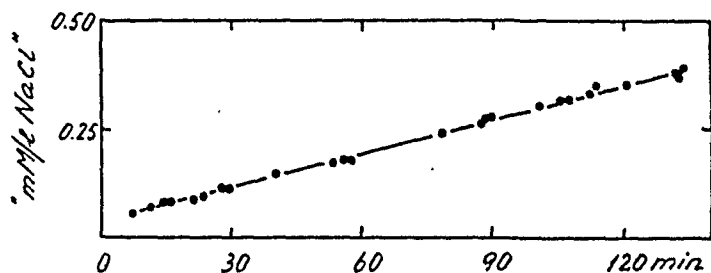


Fig. 1. "Normal" loss of salts through isolated frog skin. Physiological inside: Ringer solution. Physiological outside: Water. Abscissa: Time in minutes. Ordinate: Concentration of NaCl in mM/l.

Effect of adrenaline. Adrenaline hydrochloride (Medicinalco) added to the Ringer solution to a concentration of $1:10^6$ always produced a marked increase in the permeability of the isolated skin (fig. 2). The change in permeability sets in within the first

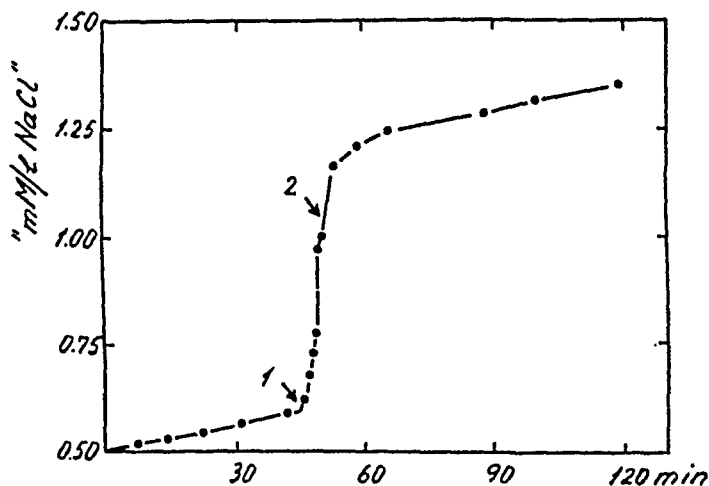


Fig. 2. Effect of adrenaline on the loss of salts through isolated frog skin. At arrow 1 adrenaline $1:10^6$ in Ringer solution. At arrow 2 Ringer.

minute after the addition of the drug. When the adrenaline containing Ringer solution is exchanged with fresh Ringer the permeability in some cases soon decreases again to the initial value.

Frequently, however, it was observed that the permeability of the skin did not resume its normal value after the Ringer solution had been replaced; in such cases normal permeability was first rapidly reestablished when the liquid in the skin pouches was exchanged. This phenomenon has some resemblance to the above mentioned high permeability of newly mounted skin, and may perhaps be due to liberation of toxins from the skin under the action of adrenaline. Adrenaline is known to induce secretion in the skin glands of anurans (WASTL, 1921). In a concentration of $1 : 10^7$ adrenaline usually produced a strong effect, as shown in fig. 3; at a concentration of $1 : 10^8$ the effect was always very weak and often completely lacking.

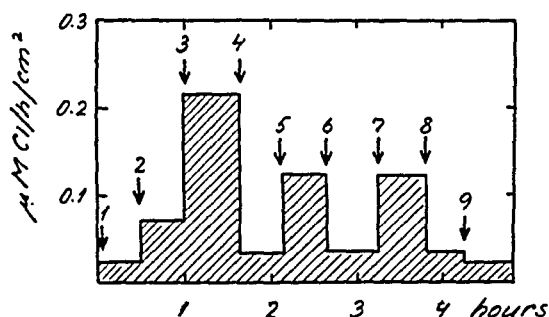


Fig. 3. Effect of adrenaline on the loss of chloride through frog skin. At arrows 1, 2, 4, 6, 8 and 9 Ringer. At arrows 3, 5 and 7 adrenaline $1 : 10^7$ in Ringer.

Effect of the red oxydation products of adrenaline (adrenochrome?).

It was observed that the action of adrenaline solutions on the permeability of the skin decreased and even disappeared when the solutions became red, even when strong solutions of adrenaline were used. In the experiment illustrated by fig. 4, the adrenaline solution ($1 : 4 \cdot 10^4$) added at arrow 2 had been prepared about an hour earlier. At the moment of addition it was already reddish. The same adrenaline solution was used again about 3 hours later (arrow 4) without producing any effect on the permeability; at that time the solution was markedly red. At arrow 5 freshly prepared adrenaline solution of the initial strength was applied for the sake of comparison, its effect thus indicating that the skin still had its normal reactivity. It is rather likely that the red colour developing during autoxidation of the adrenaline solutions is due to adrenochrome (GREEN and RICHTER, 1937, LAVOLLAY, 1943). If so the conclusion may be drawn that adrenochrome is without any effect on the permeability of the frog skin.

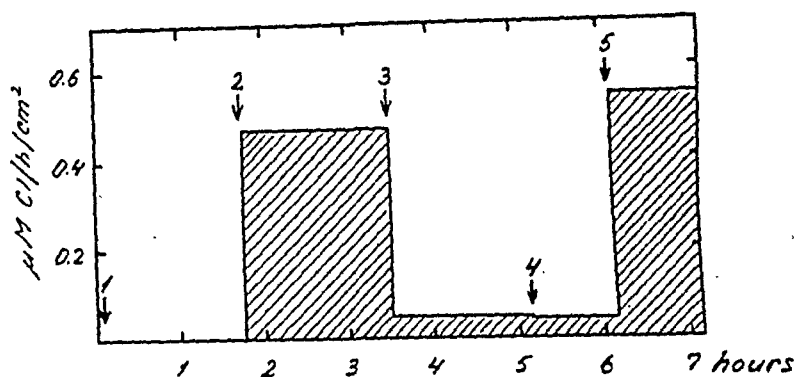


Fig. 4. Effect of red oxidation products of adrenaline (adrenochrome?) on the loss of chloride through frog skin. At arrow 1 Ringer, at arrow 2 adrenaline 1 : 4 · 10⁴ in Ringer (the solution was prepared about one hour earlier and was already reddish). At arrow 3 Ringer. At arrow 4 the same solution as used at arrow 2 — now pure red. At arrow 5 freshly prepared adrenaline 1 : 4 · 10⁴ in Ringer.

Effect of adrenoxyI. AdrenoxyI (Labaz) was used. In the light of the results obtained with the red oxidation products of adrenaline it was to be expected that adrenoxyI — the monosemicarbazone of adrenochrome — would not have any detectable effect on the skin permeability, even in concentration 1 : 10⁵ (fig. 5).

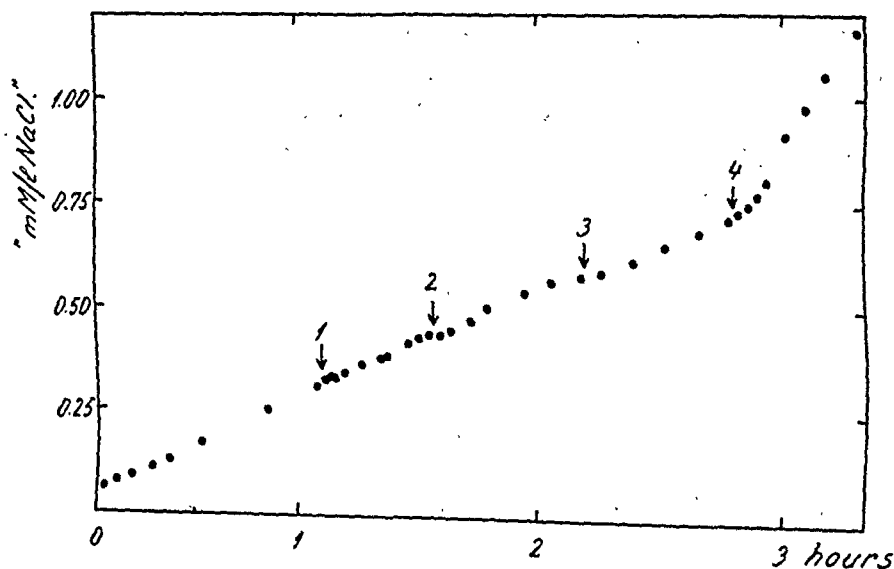


Fig. 5. Effect of adrenoxyI on the loss of salts through frog skin. At arrow 1 adrenoxyI 1 : 10⁷ in Ringer, at arrow 2 adrenoxyI 1 : 10⁵, at arrow 3 adrenoxyI 1 : 10⁵, at arrow 4 adrenaline 1 : 2 · 10⁵.

Discussion.

It has been demonstrated that adrenaline increases the permeability of isolated frog skin to ions. This effect of adrenaline on cells may perhaps prove to be of more general occurrence. HEBB and NIMMO-SMITH (1946) found that perfused isolated lungs of dogs would loose potassium to the perfusion fluid, when adrenaline was added to the latter. The same has been found on perfused isolated cat liver (D'SILVA, 1936, 1937). The effect of adrenaline on the permeability of muscles is not quite clear. STICKNEY (1940) using a preparation of frog hind limbs found that adrenaline was without any effect on the potassium exchange between the perfusion fluid and the perfused organs. On the other hand, MILLER (1943) has shown that adrenaline injected into rats has a definite effect on the exchange of ions between muscles and blood. He found the concentration of sodium to be lowered in the plasma and to be increased in the muscles as a result of the administration of adrenaline. It seems difficult to explain the findings that the concentration of potassium both in plasma and in muscles is increased under the action of adrenaline. ENGEL (1941, 1943) found an interaction between the sympathetic nervous system and the permeability of the capillaries. On cats, rabbits and dogs the permeability of the capillaries to infused Fuchsin S and sodium thiocyanate is decreased after sympathectomy, even if the capillaries are dilated as a consequence of the operation. Furthermore, the increased permeability following traumatic shock is suppressed to a great extent if sympathectomy is performed prior to the trauma. These examples support the assumption that adrenaline increases the permeability to electrolytes of a diversity of tissues.

Summary.

Isolated skin of the frog when bathed on the inner side with Ringer solution and on the outer side with water will loose salt from the Ringer solution to the water. This loss is greatly increased when adrenaline is added to the Ringer solution. Adrenaline may be active even at a concentration of about $1 : 10^8$. The red oxidation products of adrenaline (adrenochrome?) is — if at all — less active than adrenaline. Adrenoxyl in a concentration of $1 : 10^5$ is without any influence on the permeability of the skin.

The author wishes to express his sincere thanks to Professor P. BRANDT REHBERG for placing all the laboratory facilities at my disposal and for the great interest he has always taken in my work. My cordial thanks are also due to Dr. H. H. USSING for his never failing support and stimulating criticism.

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Experiments on the Primary Acidity of the Gastric Juice.

By

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Nowadays most workers in the field of mammalian gastrology believe in the Heidenhain-Pavlov's hypothesis of a high, approximately blood isotonic and constant acidity of the parietal secretion (the "primary acidity") (cf. for instance the monograph of BABKIN (1944) and HOLLANDER (1943)).¹ Much evidence has been accumulated in favour of the Pavlov view, but most of the arguments have been obtained in an *indirect* way. In 1940 one of us (T. T.) attempted a new *direct* method of determining the value of the primary acidity. The principle was based on observations that the rate of acidity reduction is proportional to the hydrogen-ion concentration of the stomach content (in accordance with the "diffusion theory", cf. TEORELL 1933, 1935, 1939, 1947). The essential part of the method was the utilization of the acid buffering power of glyccol, whereby loss of HCl owing to "back diffusion" was prevented. The results on narcotized cats gave an average value of 208 ± 6 mN HCl, a figure which was believed to be independent of the secretion rate (at least at high secretion rates).

Already in these experiments one could often record values which were considerably higher than the average, up to 310 mN, particularly at the lower rates of secretion. No significance was ascribed to these observations at that time, because the method of measuring small volume increments was regarded as less reliable.

¹ Among the very few authors who have recently questioned the hypothesis of a constant primary acidity is GUDIKSEN (1943), who, on pretty vague grounds, suggests the possibility of a limited variation.

The whole problem has now been reinvestigated with improved methods which will be described in this paper. The results obtained point towards the possibility that the primary acidity, as defined by this method, may have varying values lying between 170 mN at the higher rates of secretion and about 350, which is approached at the lower rates.

Methods.

The *general principles* of the experimental procedures were the same as described by TEORELL (1940). The new contribution to the technique consists of the addition of an *indirect* method of measuring the volume changes of the instilled stomach content. For this purpose the glycol was utilized, not only as an acidity reducing buffer substance, but also as a "dilution indicator". The previous experience (TEORELL 1939, 1940) has shown that glycol (glycine) is poorly permeable in the stomach thus making it very suitable for the purpose. Unlike many dyes which have been used by many others glycol is not adsorbed by mucus etc. The details of the new technique will be described below.

Animal procedures: In some preliminary experiments *cats* were employed, narcotized with chloralose-urethane. Each 15th minute 5 ml of 0.20 molar glycol were instilled in the emptied stomach which was ligated at the cardia and the pylorus. Hydrochloric acid secretion was induced by subcutaneous injections of ca 1 mg histamine dihydrochloride. The stomach content was completely aspirated after a 15 minutes interval and measured as to its volume directly in calibrated measuring cylinders before further analyses were begun.

The main results here presented were, however, obtained on Heidenhain pouch *dogs*. These offered several advantages over cats, in the first place because larger volumes of acid juice were produced, thus making the direct as well as indirect volume determinations more accurate, secondly because one could employ unnarcotized animals and, by administration of a continuous, intravenous injection of histamine, it was possible to maintain a desired, *constant* rate of secretion of longer time intervals (for technique see ÖBRINK 1946). Thus it was possible to get an extra check on the volume increments caused by the HCl secretion by comparison with collection periods without any instillation of a foreign liquid (cf. fig. 1). The amount of glycol solution employed was 20 ml (here 0.25 M) and the collection interval varied between 10 to 120 minutes depending on the rate of secretion.

Analyses: a) On 5.00 ml of the glycol + juice samples the *acidity* was titrated by means of N/10 NaOH using a glass electrode set up as end point indicator (pH 6.8 is a suitable end point corresponding

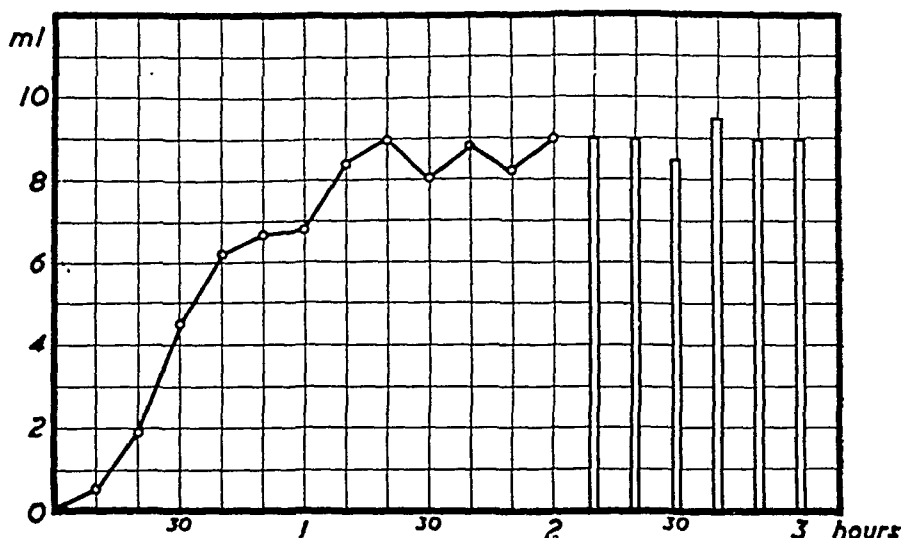


Fig. 1. The constancy of the secretion rate before (o—o—o) and during (□) instillation of glycolol. — At the time 0 injection of histamine is started. After 2 hours the first glycolol instillation is made. A typical experiment on a pouch dog.

to the value of the original glycolol solution). The NaOH consumption gives the amount of total HCl in the aliquot.

b) *Indirect volume determination*: After addition of 5 ml neutralized (to pH 9) 40 % formaldehyde the titration is continued to pH 9 and a new NaOH reading recorded (cf. Fig. 4). The last titration is evidently the well known Sørensen "formaldehyde titration" upon an amino acid, here the glycolol, and yields a value of its concentration.

After comparison with corresponding "blanks" on undiluted glycolol one can now calculate the volume increments caused by the secretion according to the following formula:

$$\text{Volume increment, } \Delta V = \left(\frac{b}{s} - 1 \right) \cdot i \dots \dots \dots (1)$$

where s = the NaOH consumption after the formaldehyde addition on the chosen aliquot (here 5 ml) of a gastric juice + glycolol sample, b = the NaOH consumption on the same volume (5 ml) undiluted glycolol ("blank") and i = the volume in ml of the glycolol instilled.

c) *The primary acidity*, i. e. the concentration of the secreted HCl referred to the volume increment, is obtained from the formula:

$$\text{Primary acidity in mN, PA} = \frac{100 a (i + \Delta V)}{f \cdot \Delta V} \dots \dots \dots (2)$$

where a = the NaOH consumption on the aliquot f (5 ml) before the formaldehyde addition, and i and ΔV retain their previous significance.

The determinations of the *total chloride* concentration were carried out electrometrically. Its primary concentration was calculated in an analogous manner as the PA.

Control Experiments.

a) *Titration controls in vitro.* "Artificial" glyocol-HCl mixtures have been titrated in the same way as described above. In order to simulate the actual experiments two different mixtures of 2.0 and 4.0 ml resp. of HCl (175 mN) added to 20 ml of glyocol (0.25 M) have been titrated. In the first case the recovery of volume gave an average of 1.97 ± 0.03 ml (one σ) and in the second one of 4.00 ± 0.06 ml (20 trials in each series).

The acidity of the increment ("the primary acidity") was determined to 177 ± 1 mN and 174 ± 1 mN resp.

The chloride determinations yielded a mean of 173 ± 6 mN.

The agreement between calculated and observed values can in all cases be regarded as satisfactory.

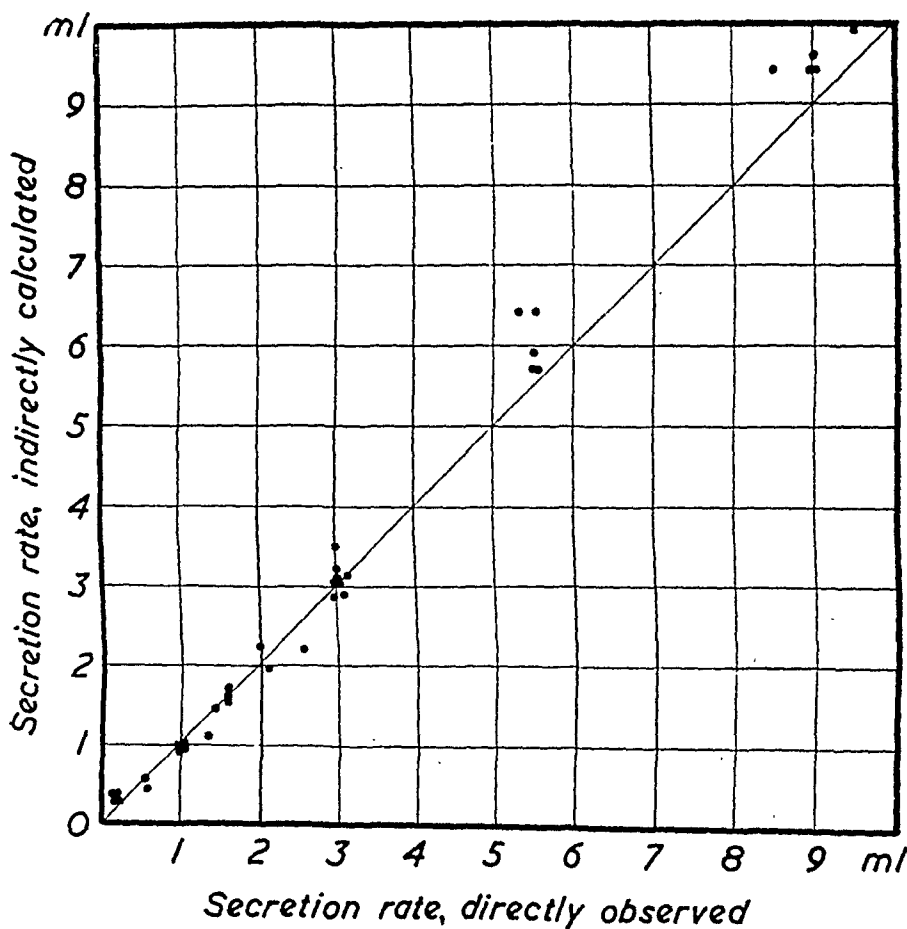


Fig. 2. The correlation *in vivo* between the secretion rate (ml/10 min.) directly observed and indirectly calculated according to formula (1).

b) *Recovery controls of the volume in vivo.* The accuracy of the constant secretion on the dogs was within 3—4 % of the mean over a time range of half an hour. This constancy was maintained before as well as during the periods of instillation of glyocol (cf. fig. 1). The agreement between the volume increments directly observed in a measuring cylinder and those indirectly obtained by the formula (1) is shown by the fig. 2. This figure indicates a good agreement between the two methods. It is thus stated that *glyocol may be used as a reliable dilution indicator* of the stomach secretion.

Results.

a) *Cats:* First a typical result obtained on a whole stomach from one *cat* experiment will be mentioned (Table I).

Table I.

A cat experiment showing the volume increment (ΔV) and the primary acidity (PA).

Time (min.)	ΔV (ml)		PA (mN)		Neutral Cl in sample (mN)
	dir.	indir.	dir.	indir.	
0—15.....	5.1	5.7	167	189	24
15—30.....	7.5	6.2	180	195	21
30—45.....	6.6	5.9	197	206	17
45—60.....	4.8	4.5	224	234	16
60—75.....	2.9	2.9	244	244	17
75—90.....	1.5	1.7	298	267	15
90—105.....	0.8	1.1	342	288	18
105—120.....	0.6	0.8	386	308	18

The table demonstrates clearly that the primary acidity values become surprisingly high towards lower rates of secretion. The neutral chloride excess of the samples seem to remain approximately constant.

Although the same type of result has been found on other cat experiments, we have thought it advisable to postpone any discussion of the possible significance of our figures until further experience has been gained on *dog* pouches which as mentioned previously, offer several advantages. Such experiments will be reported below.

b) *Dogs:* A typical result on a *dog* pouch is shown in the fig. 1. When the secretion rate is constant, 20 ml glyocol were instilled and withdrawn every 10 minutes. The observed volume incre-

ments are indicated by columns which are seen to be almost equal to the secretion rate observed before the instillation, hence it can be concluded that the glycol does not interfere with HCl-production, nor does it cause any appreciable "osmotical" water transport. No correction terms are thus necessary. In Table II the total body of experimental figures is given for one 20 kg dog. The relation between directly observed and indirectly calculated secretion rates (i. e. the volume of juice produced in 10 minutes) has already been presented in the fig. 2.

Table II.

Survey of the experimental data (dog).

ΔV ml		Observation time (minutes)	q (ml/10 min.)		PA (mN)		Total Cl (mN)		Neutral Cl (mN)		pH of the sample
dir.	indir.		dir.	indir.	dir.	indir.	dir.	indir.	dir.	indir.	
0.8	2.4	60	0.13	0.40	318	114					
3.1	4.8	180	0.17	0.26	159	112					
0.5	0.8	30	0.17	0.27	210	133					
0.5	1.2	30	0.17	0.40	151	65					
7.0	6.5	120	0.58	0.54	330	349	359	379	29	30	3.0
3.0	2.4	50	0.60	0.48	302	354	307	373	5	19	3.2
5.7	6.0	60	0.95	1.00	299	287	315	303	16	16	2.4
6.0	5.9	60	1.00	0.98	270	274	290	294	20	20	2.4
6.0	5.6	60	1.00	0.93	268	282	312	329	44	47	2.6
6.0	5.9	60	1.00	0.98	278	282	303	307	25	25	2.6
8.2	6.6	60	1.37	1.10	219	256	268	315	49	59	3.3
8.6	8.5	60	1.43	1.42	225	227	226	228	1	1	3.2
9.5	9.6	60	1.60	1.60	212	211	314	311	102	100	2.9
9.6	9.4	60	1.60	1.56	222	225	240	244	18	19	2.7
9.6	10.1	60	1.60	1.70	230	222	240	232	10	10	3.0
2.0	2.2	10	2.00	2.22	216	198	297	272	81	74	3.3
5.3	4.9	25	2.12	1.96	210	214	277	295	67	81	3.0
3.8	3.3	15	2.53	2.20	214	242	263	297	49	55	3.2
5.9	5.7	20	2.95	2.85	186	190	206	212	20	22	2.9
6.0	6.2	20	3.00	3.10	184	179	221	216	37	37	2.9
6.0	6.2	20	3.00	3.10	181	183	186	182	5	—1	3.0
6.0	6.0	20	3.00	3.00	190	190	195	195	5	5	2.9
3.0	3.2	10	3.00	3.20	191	180	260	246	69	66	3.0
3.0	3.5	10	3.00	3.50	214	188	246	215	32	27	2.9
6.2	5.8	20	3.10	2.90	190	192	224	236	34	44	2.8
6.3	6.2	20	3.15	3.10	187	189	202	206	15	17	2.9
5.3	6.4	10	5.30	6.40	175	152	205	177	30	25	3.0
5.5	6.4	10	5.50	6.40	180	160	199	177	19	17	2.9
5.5	5.9	10	5.50	5.90	179	170	209	198	30	28	3.0
5.5	5.7	10	5.50	5.70	172	168	199	194	27	26	3.0
5.5	5.7	10	5.50	5.70	175	170	199	194	24	24	3.0
8.5	9.4	10	8.50	9.40	188	169	194	181	6	12	2.5
9.0	9.4	10	9.00	9.40	173	168	174	169	1	1	2.8
9.0	9.4	10	9.00	9.40	173	170	187	181	14	11	2.7
9.0	9.6	10	9.00	9.60	181	173	187	179	6	6	2.6
9.5	9.9	10	9.50	9.90	175	170	195	185	20	15	2.8

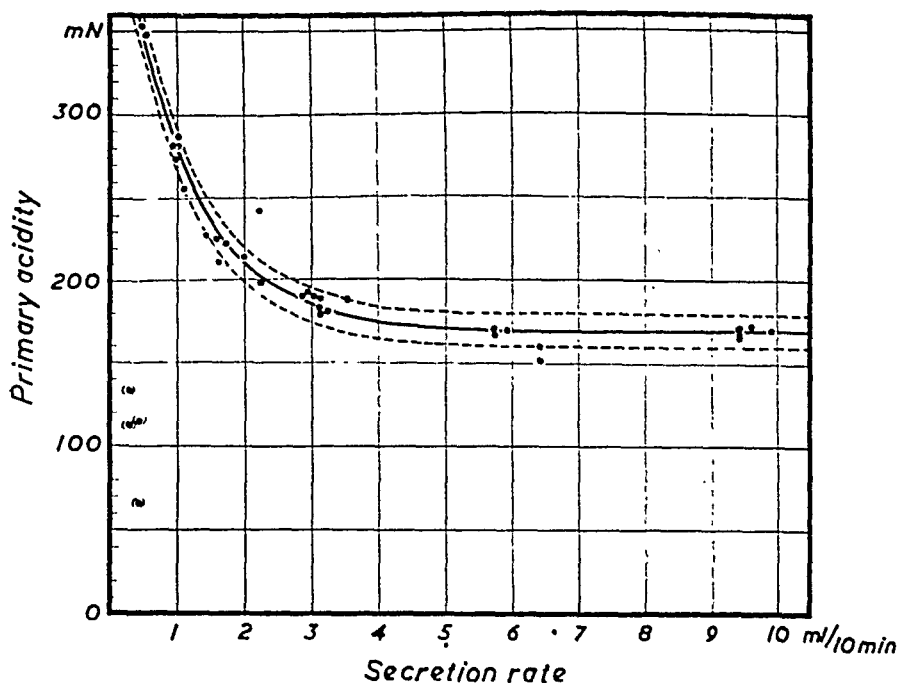


Fig. 3. *The influence of the secretion rate upon the primary acidity.* — The observed values are smoothed according to a simple exponential function (full line). The index of correlation is 0.964 ± 0.006 . The standard error of estimate is 10^1 (dotted lines).

In a separate graph (Fig. 3) we have plotted the *primary acidity* calculated from formula (2) as a function of varying rates of secretion. The ΔV employed were those obtained by the indirect "glyocol dilution method", the directly determined ΔV :s yield almost exactly the same curve. The curve was smoothed according to the following purely empirical equation $PA = PA_{\infty} + (PA_0 - PA_{\infty}) \cdot e^{-cq}$ where the indexed PA :s denote the primary acidity at a secretion rate of zero and infinite respectively, $e = 2.71$, $q =$ the secretion rate (ml/10') and c a constant. The material comprises 32 different determinations on the same dog. By means of the method of least squares the following numerical equation was found to give the best fit: $PA = 169 + (464 - 169) \cdot e^{-1.0q}$. This means that the highest primary acidity could be extrapolated to 464 mN at the lowest possible rate of secretion, at higher rates it gradually approached the value of 169 mN as a lower limit. The statistical limits of error are indicated in the figures as dotted lines. At extremely low secretion

¹ For terminology see: M. EZERIEL, *Methods of Correlation Analysis*, New York, 1941.

rates, where the collection has been extended over longer time periods (up to 2 hours) there is a marked tendency to a decline in the primary acidity. These figures, put within brackets in the graph, have been excluded as "abnormal", because it seems probable that even minute amounts of non-acid secretion here exert an influence.

The *total chloride* values follow the primary acidity values very closely, but they are, in general, somewhat higher (about 20 mN in 10 minute samples).

It is worth noticing that the hydrogen ion concentration of the glyocol juice samples at their withdrawal never was higher than $10^{-2.4}$ N ($\text{pH} \geq 2.4$). This is less than $1/10$ of the hydrogen ion concentration of pure gastric juice, which ought to diminish the loss of H^+ ions due to diffusion to a corresponding fraction, i. e. from a practical point of view diffusion loss may be negligible.

In some experiments a phosphate buffer has been tried as an acidity reducing agent. The results showed a trend similar to the one reported above on glyocol.

Finally it should be remarked that the glyocol-juice samples all were colorless and clear and contained very small amounts of visible mucus.

Discussions.

Both in experiments on whole cats' stomachs and, in particular, on dog's stomach pouches it has been found that the so called primary acidity (PA), i. e. the calculated acidity of the parietal secretion "in statu nascendi", obtains values which, in contrast to our preliminary belief, was not at all constant. Although values of the commonly adopted PA value of ca 175 mN HCl (i. e. near blood isotonicity) could be recorded in a great deal of our experiments, it was often found that values *exceeding* 175 mN could be registered. A distinct dependence on the rate of secretion as to the PA was also evident: the *lower* the rate of secretion the higher the PA, which approached about the double conventional value at the lowest rates (*extrapolated* maximal PA was 464 mN). This is a most surprising result and certainly calls for a thorough discussion as to possibilities of experimental errors etc. before any physiological significance can be attached to it.

In the first place one has to consider the reliability of the experimentally determined quantities, i. e. the volume increments and the figures obtained at the NaOH titrations. In the second

place one has to investigate the possibility of "artefact" results due to the special buffer technique employed.

As to the volume increments there is evidence indicating that they are not far from correct: 1. In the experiments with constant rates of secretion (with the continuous, constant histamine injection) the actual output of the secretion is practically the same as the values measured after periods of glyocol instillation (cf. the figure 1). 2. The *in vitro* model experiments yielded almost the calculated volume values. 3. The direct volume measurements agree satisfactorily with those determined indirectly according to the "formol titration method" also in the actual *animal* experiments.

As regards the NaOH consumption one might expect that the admixture to the stomach content of a component other than the HCl with a high base binding capacity could result in too high a NaOH titration figure and thus cause excessively high PA values when inserted in the formula (2). Now, it seems reasonable to assume that such a postulated component should not be a strong acid. If it was a weak acid or an ampholyte (protein, mucus) a considerable base binding ought to show up in the pH range of 4.0—6.8. When one plots the pH as a function of the NaOH consumption in a usual "titration curve", there ought to appear definite "steps" or inclined slopes. Instead special investigations on a number of our samples showed that the graphs invariably were perfectly flat in this critical pH region (as 2 in fig. 4). Hence, we are forced to conclude that no weak acids or acid ampholytes can account for the high NaOH figures obtained. It might perhaps be questioned whether Na-bicarbonate could be present and yield an excess of liberated CO₂ which could remain dissolved even in the acid samples (pH about 2—3) and then be subject to "back titration" with NaOH. In such an event, however, a marked S-shaped bend should have been discovered on the titration curve in the region 4.5—6.8 (pK for H₂CO₃ is 6.1), cf. (3) in fig. 4. In spite of special precautions we have never found such an indication of the presence of CO₂. Incidentally it may be mentioned that the CO₂-production of the stomach, if anything, is diminished in the presence of acid content (cf. TEORELL 1933 p. 264).

As the considerations above seem to allow an exclusion of purely analytical errors as sources of the unexpectedly high PA:s, it remains to examine the possibilities of "artefacts" resulting from

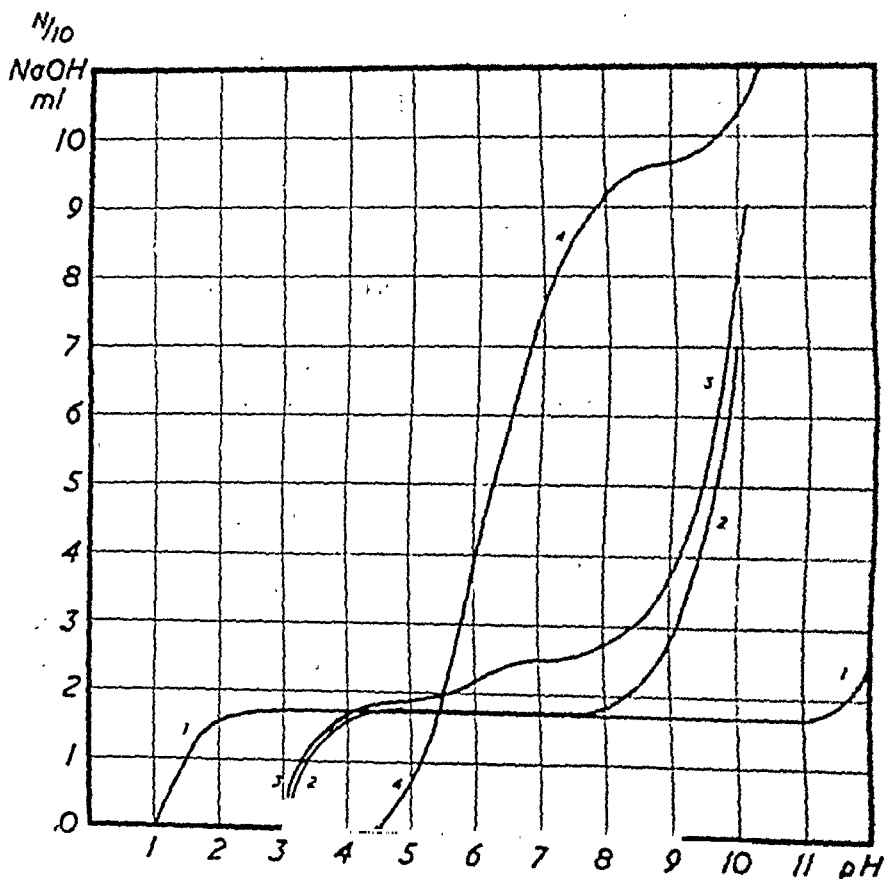


Fig. 4. Titration curves (with continuous stirring, in air): — (1). 1 ml 175 mN HCl. — (2). 1 ml 175 mN HCl + 4 ml approx. 0.25 M glycolol. — (3). 4 ml approx. 0.25 M glycolol + 0.015 g NaHCO_3 (i. e. equivalent to 1 ml 175 mN H_2CO_3) + 1 ml 350 mN HCl. — (4). 4 ml approx. 0.25 M glycolol + 5 ml 40 % formaldehyde (neutralized to pH 9.0).

the special collecting conditions which have been employed. Briefly stated, if one postulates that a PA of ca 175 mN is the actual figure, the high PA:s values > 175 ml should be due to an excess of HCl equivalents having entered the gastric content without a corresponding volume increment. The question thus arises, from where do these extra HCl equivalents come? This question can not be satisfactorily answered with the data at hand, but for the sake of discussion some possibilities will be mentioned. It can be imagined that the postulated, almost "tissue isotonic" parietal secretion of 175 mN strength of free HCl is the results of some kind of chemical or physico-chemical equilibrium prevailing in site of formation of the HCl or in ducts in the mucosa which lead off the secretion outwardly. If the glycolol buffer could

penetrate as deep as to these places the H^+ -ion concentration will be diminished there and, hence, the postulated equilibrium disturbed, whereby new HCl should be produced as a "compensation" mechanism. Such a penetration of glycol ought to be less marked at the higher rates of outflow of the parietal secretion, which acts as a counter stream in opposition to the penetration direction of the glycol molecules. Hence, more "normal" conditions would prevail at the higher rates of secretion. At the lower rates, however, the "intraglandular" neutralization would cause a take up of excess HCl from its formation sites (the parietal cells or cell surfaces?). Unfortunately this "explanation" is a pure product of speculation, because no experimental evidence is at present available to speak for or against it.

The same can be said of a suggestion that the excessive accumulation of HCl should be due to a phenomenon of diffusion. If some fraction of the parietal secretion should remain in the "crypts", and the mucosa surface (possibly with its mucous lining) acting like a permeable membrane, the HCl would *diffuse* to the glycol volume. In such a case the diffusion would yield an approximately constant contribution of HCl to the glycol of the stomach content and thus be of a greater importance at the lower secretion rates.¹

It is worth noticing that glycol also can give rise to "accelerated diffusion" or "diffusion effects" of the type described by TEORELL (1935, 1937). In model experiments, for instance, it can be readily demonstrated that the transport of HCl across a diffusion membrane takes place at a higher rate in a system of the type (outside) *HCl* (membrane) *glycol* (inside) than in a system *HCl* (membrane) *NaCl*. The amount of titrable acidity (to pH 6.8) can, at least temporarily, reach values more than 50 per cent in excess of that of the pure HCl solution. An analogy to such a system might perhaps be imagined in the living stomach. Thus the crypts will be drained on HCl without much volume change. Although such mechanisms could be valid for a glycol instillation period immediately following an (abrupt) cessation of the parietal secretion, they seem to be hard to apply during periods of continuous acid production.

In conclusion it may be said that our attempts to scrutinize the experimental conditions for possible pit falls as to the existence of "artefacts" have failed so far. It leaves us with the

¹ In this connection it should be pointed out that the slight excess of total-Cl over the acidity (in Table I and II denoted as "neutral chloride" and nearly constant) can be accounted for by assuming some alkali chloride diffusion from the stomach wall to the stomach content (cf. several graphs TEORELL (1939)).

possibility that the primary acidity of the parietal secretion actually can be *variable* and *much higher* than generally believed hitherto, *lying in the range 170—350 mN*. If these results be true it may open new points of attacks on a central problem in stomach physiology, the mechanism of formation of the hydrochloric acid, which is still completely unsettled.

Summary.

1. The problem of the magnitude of the "primary acidity" of the parietal secretion of the stomach has been reinvestigated with a new, improved method. Its essential part consists in the utilization of the acid buffering power of glycol combined with the employment of that substance as a "volume indicator". With this method the acidity reducing diffusion processes are abolished and reliable volume measurements can also be carried out.

2. Experiments have been performed on cats and, in the main, on Heidenhain pouch dogs. In the latter case continuous, intravenous histamine injection has been used as a gastric acid stimulant which yields constant, controllable outputs of HCl secretion.

3. The primary acidity (found by dividing the amount of titrable acid by the volume increment of an instilled, "isotonic" glycol solution) has now been found to be variable and can be unexpectedly high. It ranges between 170 and about 350 mN. There is a definite relationship between the primary acidity value and the rate of secretion: The lower the secretion rate, the higher is the primary acidity.

4. The significance of the findings are discussed.

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Our thanks are also due to Miss M. UHRSTRÖM for valuable technical assistance.

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The Importance of the Stalk Connexion for the Power of the Anterior Pituitary of the Rat to React Structurally upon Ceasing Thyroid Function.

By

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The problem whether the pituitary body is able to retain its incretory activity independent of direct impulses from the mesencephalic centres has been the subject of a large number of investigations. In the majority of these a study has been made of the power of transplanted hypophyseal tissue to stimulate endocrine organs whose functions are dependent on the pituitary body. Most of the investigators find that hypophyseal transplants, contrary to transplants from other endocrine organs, show no power to retain their incretory function. There are, however, opinions that diverge from this view. The divergence in views may be due in part to differences in absorption from the hormone depot supplied with the transplant and in part to the employment of different criteria for the evaluation of the activity in the endocrine glands stimulated by the transplant.

The significance of the hypothalamus for the hypophyseal function has also been studied after severance of the stalk connexion with the infundibulum. Such a procedure would no doubt be best suited to the problem under consideration, but on account of the technical difficulties it is less utilized than transplantation experiments. These latter experiments have not been claimed to bring any advantage in other respects. When the pituitary is left *in situ*

with an unbroken stalk connexion, the chief advantage gained is that all the blood vessels not having their course in the stalk are left intact.

With the pituitary body *in situ*, the activity of the hypothalamus has been most thoroughly studied with respect to the gonadotropic function. In a series of experiments on the rabbit in 1937 and later on both the rabbit and rat WESTMAN and JACOBSON found that the gonads showed regressive morphological changes and reduced function after stalk section. These results have been confirmed by some investigators but have been rejected by others. The literature on the subject has been recently reviewed by BROLIN (1945). Reliable experimental results would seem only to have been obtained when, in addition to the stalk connexion with the posterior lobe of the pituitary gland, the direct connexion with the anterior lobe via the pars tuberalis has also been broken. After such a complete severance of the stalk it has been made clear that the gonadotropic function of the hypophyseal anterior lobe is powerfully reduced.

The importance of the stalk connexion for the *thyrotropic function* of the anterior lobe has been studied by different methods. First it may be mentioned that the functional state of the thyroid gland after section of the hypophyseal stalk has been studied by several investigators. In the rabbit BROOK (1938) found normal thyroid gland after the operation. WESTMAN and JACOBSON (1942), likewise in the rabbit, found normal thyroid glands in some cases and in a somewhat larger number of cases glands showing signs of reduced function. Working on the rat, WESTMAN and JACOBSON (1938) came to a similar result and pointed out that where regressive thyroid changes are observable these are not so pronounced as after hypophysectomy. UOTILA (1939) states that the structure of the thyroid gland remains unchanged after the operation. On the other hand, BROLIN (1945) found that after complete stalk section the thyroid activity in the rat is reduced and that this could not depend upon chance. Concordant experimental results obtained by UOTILA (1939) and BROLIN (1945) show that the increase in thyroid activity which arises in cold-exposed rats does not appear after section of the pituitary stalk. When complete transection of the stalk had been established, BROLIN (1945) found that stalk-cut rats had a low resistance to cold. Many of these animals, however, could endure a continuous stay of four to five weeks in a low environmental temperature. Hence, a limited power to resist the effects of a low outdoor temperature persisted after complete stalk transection, which, as known, is not the case

after hypophysectomy. This observation does not seem to prevent the cited experimental results from being interpreted to the effect that the thyrotropic function of the pituitary body is highly dependent on the stalk connexion. A view incompatible with this interpretation is that advanced by UOTILA (1940) and reproduced here by the following quotation. "It is concluded that the thyrotropic function of the anterior hypophysis has a basic secretory rhythm, which is for the most part controlled humorally by variations in the organisms thyroxine level without the mediation of the hypothalamo-hypophyseal pathways." UOTILA bases his opinion on two groups of experiments in which rats with sectioned hypophyseal stalks were examined. In one group he found that injections of thyroxine reduced the thyroid activity as much in these rats as in the intact ones. In the other group he observed that residual thyroid tissue exhibited a compensatory hyperplasia in the stalk-sectioned rats as well. The evidential value of the first-mentioned results have been undermined by the statement that stalk section alone involves a reduced thyroid function, as was shown later by BROLIN (1945). Moreover, WESTMAN, JACOBSON and HILLARP (1943) as well as BROLIN (1943) have found that the completeness of the stalk severance in UOTILA's series of experiments is open to question.

In the investigations on the rôle played by the hypothalamus for the thyrotropic function of the hypophysis the functional state of the thyroid gland has been mainly treated. The kernel of the problem, which lies in the reactions of the anterior pituitary, is then only indirectly illuminated.

The possibilities of interpreting the cytological reactions involved in the production of the thyrotropic hormone have been studied by BROLIN (1945). In conjunction with quantitative and qualitative morphological analyses of the anterior pituitary experiments were carried out with cold exposure, thyroxine injections and thyroidectomy as well as hormone analyses. The results afforded very strong reasons for the conclusion that thyrotropic hormone is produced in the basophil cells. In these, an active productive phase and a degenerative phase could be observed. The latter was represented in the final stages of the thyroidectomy-cells and identical cell-forms as arise in cold.

Among the investigations undertaken to elucidate the connexion between the cellular structure and the hormone production SMELSER's (1944) exhaustive quantitative determinations of the topographical

localization of the hormones in the anterior hypophysis deserve mention. Starting from the known fact that the hypophysis of the ox consists of different zones with differences in cell distribution and hormone occurrence, he calculated the concentration of the hormones as a "center cortex ratio". Among other things he found that the basophile-rich central part contained double as much gonadotropic hormone as the cortical part but 16 to 32 times as much thyrotropic hormone and 6 to 8 times as much adrenotropic hormone. Without drawing any definite conclusion he considered the result to suggest that these three hormones "are produced or stored by several distinct cell types which have different special distributions in the gland . . .". This standpoint is not incompatible with the view that basophil cells are producers of the gonadotropic FSH factor as well as of the thyrotropic hormone, for different types of basophiles with hitherto uninvestigated structural differences may exist. However, there is also another possible interpretation that must not be overlooked, since it is conceivable that micromorphologically identical basophiles may produce the two hormones in different amounts in response to different impulses. The topographical course of nerves or possibly of vessels from different hypothalamic centres might then explain differences in the hormone distribution, since the basophilic cells do not only occur in the central portion of the anterior lobe. Hence, SMELSER's experimental results ought not to be considered to conflict with the view that the hormone production of the anterior lobe can to some extent be cytologically localized. Instead, they accentuate the question whether the same cells produce more than one hormone, or whether the known types of cells can be structurally differentiated to the same extent as different anterior lobe hormones can be distinguished. Whatever critical shape the interpretations of SMELSER's results may take, it can ultimately be concluded that the 16 to 32 times greater thyrotropic hormone content of the basophile-rich zone does not contradict the interpretation that these cells produce thyrotropic hormone.

The investigations hitherto carried out show that the thyrotropic function of the pituitary body can be influenced by the latter's stalk connexion with the hypothalamus. As to whether the anterior pituitary-thyroid system can function in its essential parts autonomously there is a divergence of opinions between different investigators. An investigation of the cell reactions of the anterior lobe after severance of the stalk connexion attacks the heart of this question and can therefore contribute to its solution.

When the significance of the pituitary stalk for the function of the anterior lobe is being studied, it should be remembered that the stalk is not a uniform anatomical structure. Regard should therefore be had to both the *pars infundibulis* and the *pars tuberalis*, as the last-mentioned glandular part contains nerve-bundles and vessels that form a direct connexion between the

anterior pituitary and the hypothalamus. This system of innervation has been studied especially thoroughly in the rat by HILLARP and JACOBSON (1943).

Experimental Procedure and Material.

The underlying principle of the experiments is to put the experimental animals in such a situation that an increased thyroid activity is demanded. Thyroidectomy has been employed for this purpose because of the extensive and distinct qualitative changes it brings about in the basophile cells. This method, moreover, is reliable not only on account of the distinctness of the cytological reactions arising but because it gives the assurance that reactions will not fail on occasion to appear. Thyroidectomy has on many occasions, and sometimes to a great extent, been performed by the same technic on rats belonging to the stock kept by me for several years. It was found without exception that the animals exhibited distinct changes in the basophil cells under the development of thyroidectomy cells. In conjunction with the experiments carried out in this series, therefore, it was only necessary to carry out thyroidectomy in a limited number of cases in order to establish the constancy and reliability of the cytological changes.

When thyroidectomy was undertaken, the parathyroid glands were left *in situ* in accordance with a procedure previously described (BROLIN, 1945). The parathyroid glands left in position were microscopically examined at the autopsy undertaken.

The pituitary stalk was cut off under ocular control by a method devised by me (BROLIN, 1943). In the planning of the experiments care was taken not only to break off the entire stalk connexion but also, in some cases, to sever one of these two components selectively. It was possible to realize this wish so far as the *pars infundibulis* was concerned. On this account the experiments have been divided into two groups: series A including experiments in which the pituitary stalk was severed and B experiments in which the intermediate and posterior lobes were extirpated. The distribution of the material is shown in Table 1. According to a method described by SMITH (1931), that part of the processus infundibuli which consists of the posterior lobe and with it the intermediate lobe was removed in one piece. By this means the influence of these two lobes and of the nervous fibres running through them was eliminated. The extirpation was carried out by means of a thin glass cannula in which a controllable pressure was established maintained below atmospheric. Regulation of the suction power was simplified by means of a three-way tube, which was connected to the cannula, a water-suction, and a rubber tube which the operator grips between his teeth. The pressure in the cannula is dependent on the inflow of air through the rubber tube, the lumen of which can be readily altered by means of the operator's teeth.

The organs examined were fixed in Susa immediately after the rats had been killed by clipping their cervical vessels under ether

anaesthesia. With carbon tetrachloride and carbon bisulphide as intermedia the organs were thereupon embedded in paraffin, after which they were sliced with a sledge microtome. In that part of the investigation in which the stalk was cut a coherent block of hypophysis and mesencephalon was cut into series of $8\ \mu$ sagittal slices. This enabled the effect of the operation on the stalk to be controlled. The same technique was partially used in the experimental series in which the intermediate and posterior lobes were extirpated. In some remaining cases the pituitary body was prepared separately and sliced into $4\ \mu$ horizontal sections. Sections from all the pituitaries were stained by MARTIN'S method (1933). In the stalk-sectioned series azan staining was in addition carried out on sections preserved separately for the purpose. In the second series azan staining was also employed in some cases parallel with MARTIN'S.

Besides the pituitary body, the parathyroid glands, gonads and adrenals left in position at the operation were microscopically examined. The testicles were stained with ferric alum hamaetoxylin and benzo-purpurin, the other organs with EHRLICH'S haematoxylin as nuclear stain and eosin or chromotrop 2 R as plasmic stain.

The material. As already mentioned, there were two experimental series. In one, series A, the severance of the pituitary stalk was followed later on by thyroidectomy, and in the other, series B, extirpation of the posterior and intermediate lobes of the pituitary was likewise followed by thyroidectomy. In both the series intact and thyroidectomized controls were taken from the same litter. For this purpose litters in which the majority of the individuals belonged to one sex were chosen, and only these individuals were employed. In a couple of large litters, however, both sexes were included.

The rats were fed the whole time with grain, boiled potatoes, milk and lungs which were available under the rationing regulations in force. All the rats included in the investigation, viz. 45, were well developed and free of defects from the point of view of condition. When the rats were about 7 months old, the experiments were started by severing the pituitary stalk in series A and by extirpation of the intermediate and posterior lobes in series B. About one month later some of the rats operated upon and some of the control animals previously unoperated upon were thyroidectomized. After another $1\frac{1}{2}$ to 2 months the animals were killed.

Results.

A. Experiments with Severance of the Pituitary Stalk.

The six intact animals used as controls showed in all respects normal qualitative cell conditions in the anterior pituitary. Their cell-picture entirely agreed with the observations made on a couple of hundred normal individuals belonging to the strain of animals used.

The anterior lobe may be considered to be built up of two rather large lateral parts that hang together by a small isthmus below and behind the intermediate lobe. No differences between the two lateral parts were observed.

In six cases thyroidectomy was carried out to verify that hypertrophy and vacuolation had arisen in the basophil cells. As on other occasions, typical cell reactions that were clearly visible in the two lateral parts of the anterior lobe were also obtained this time.

In six cases the pituitary stalk was cut off without any further operation being made. The stalk severance was complete in four cases, but in the two other cases it was incomplete. In those cases in which the pituitary body was completely severed the stock of cells had altered in the direction of a more or less pronounced uniformity. For instance, the chromophil cells were so degranulated and diminished that the cell-picture was dominated by small cells of similar form, which could most closely be classified as chromophobic.

A narrow *pars tuberalis* connexion with one of the lateral parts of the anterior lobe showed incomplete stalk section in one case. This lateral part with direct stalk connexion presented a normal cell-picture, whereas the other lateral part devoid of direct connexion showed a uniform cell-picture. The other case with partial stalk connexion exhibited a rather large intact portion of the stalk and a normal cell-picture.

In the eight cases in which section of the pituitary stalk was later followed by thyroidectomy a complete break in the continuity was observed in three cases and a unilateral one in four cases. Thus, in these four cases one lateral part of the anterior lobe was without direct stalk connexion, while its other lateral part was in immediate connexion with the hypothalamus by a narrow lateral piece of the *pars tuberalis*. The remaining eighth case exhibited a partial stalk break with a persisting relatively broad stalk connexion intact.

When the break in the continuity of the stalk was complete there were no thyroidectomy cells. Further, a more or less pronounced uniform cell-picture was observed such as in stalk section not followed by thyroidectomy.

Severance of the direct stalk connexion with one of the lateral parts of the anterior lobe and remaining contact between *pars tuberalis* and the other enables one of the lateral parts to be used

as a control when cell-changes are being studied in the other. In the four cases in question the lateral part furnished with a *pars tuberalis* connexion showed typical cell-changes with hypertrophized and vacuolized basophil cells, while the other lateral part presented considerably less pronounced changes or a uniform cell-picture (Fig. 1). In the previously mentioned eighth case with a persisting wide stalk connexion there were typical cell alterations in both the lateral parts of the anterior lobe after thyroidectomy.

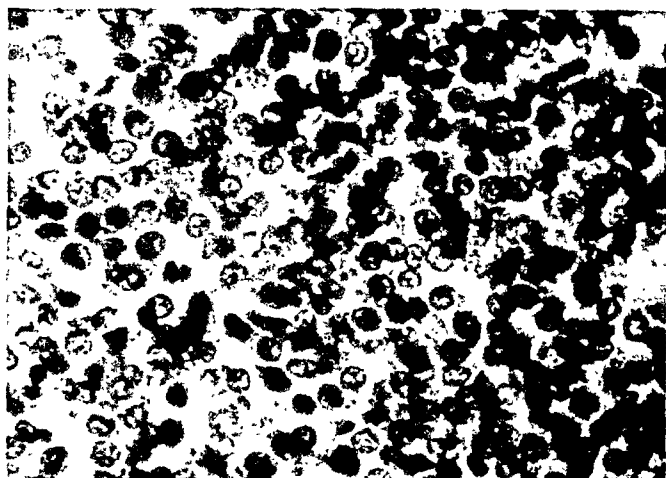


Fig. 1. Uniform cell-picture in one of the two lateral parts of the anterior pituitary, the corresponding *pars — tuberalis —* connexion having been severed. No reactions of the basophile cells are discernible in spite of performed thyroidectomy.
× 500.

The results of the experiments in series A show that when the pituitary stalk including the pars tuberalis is severed hypertrophy and vacuolation are prevented from arising in the basophil cells after thyroidectomy.

B. Experiments with Extirpation of the Intermediate and Posterior Lobes of the Pituitary Body.

In two intact and in three thyroidectomized rats the pituitary cell-picture agreed entirely with what had been observed in this material of animals.

The intermediate and posterior lobes of the pituitary were extirpated *en bloc* in six rats and no remains of the extirpated lobes could be discovered on the serially sliced pituitary preparations in any case. The cell-picture of the anterior lobes left *in situ* showed no deviation from normal conditions.

In eight cases extirpations of the intermediate and posterior pituitary lobes in one piece was followed later on by thyroidectomy. Only in one of the serially sectioned pituitary bodies could the extirpation be denoted as incomplete, a small inconsiderable group of intermediate lobe cells that had been left behind being observed here, whereas no traces of the posterior lobe could be discovered. The eight anterior lobes left *in situ* showed the typical post-thyroidectomy cell-changes in the form of hypertrophized and vacuolized basophil cells (Fig. 2).

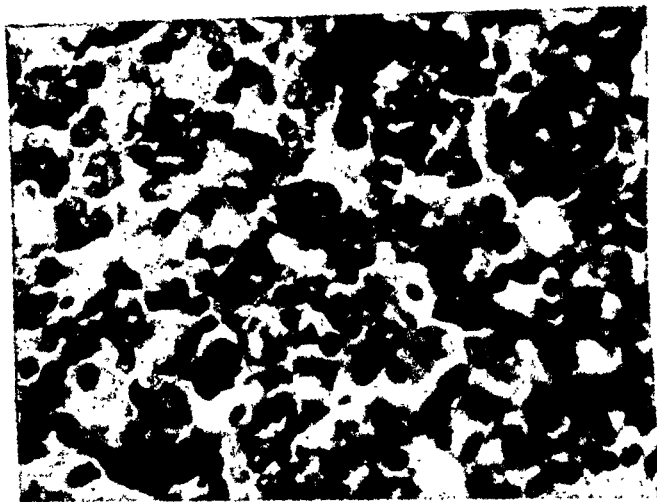


Fig. 2. Several enlarged and some vacuolated basophile cells in the anterior pituitary after thyroidectomy. The posterior and intermediate lobes have before then been extirpated, yet the cell-changes have independently developed.
× 500.

After extirpation of the two other lobes the anterior lobe still retains connexion with the *pars tuberalis* of the stalk, which could be observed in the preparations in which the hypophysis and hypothalamus had been cut out *en bloc*. From the results obtained in experimental series A it was likewise evident that a single remaining *pars tuberalis* connexion is alone sufficient for typical cell-changes to arise after thyroidectomy.

The experiments carried out show that when the anterior lobe is in connexion with the pars tuberalis of the stalk typical cell-changes arise after thyroidectomy independently of the intermediate lobe and the posterior lobe and the nerve fibres coursing them.

On examination of those organs which, besides the pituitary, had been preserved it was found that microscopically normal parathyroid tissue could be observed after thyroidectomy in almost all cases. Consistent with what was previously known, signs of atrophy

could be demonstrated without exception in the gonads after complete stalk section, which was not the case after extirpation of the intermediate and posterior lobes or when there was a remaining *pars tuberalis* connexion.

Table 1.

Survey showing use of the material.

Series A. Experiments with section of the pituitary stalk		Series B. Experiments with extirpation of the middle and posterior lobes of the pituitary	
Operation	No.	Operation	No.
Stalk section-thyroidectomy	8	Extirp. of middle & post. lobe-thyroidectomy	8
Stalk section	6	Extirp. of middle and post. lobes	6
Thyroidectomy	6	Thyroidectomy	3
Controls not operated on	6	Controls not operated on	2

Discussion.

Direct operation on the pituitary stalk showed in seven cases of eight that the response of the basophil cells to thyroidectomy was dependent on the stalk connexion. As this connexion had been completely broken off in three of these eight cases, basophil cell reactions were entirely precluded. As this part of the material is not large, it may be questioned whether chance factors have exercised any influence. Here, however, it is a question of distinctly observable obvious cell-reactions which arise obligatorily. Moreover, their occurrence in the material used has been established. Even if the chance of cell-reactions not appearing be set so high as one in ten, the probability of their non-appearance owing to chance-factors in three cases is only one in a thousand. However, when judging the results of this mainly qualitative study account should be chiefly taken of systematic factors. The chief source of systematic error consists, as was pointed out, in inadequate stalk operations, but this source would seem to have been eliminated in the present cases. Another important question is whether the time interval between stalk section and thyroidectomy was made sufficiently long, for when vessels of the stalk are torn away the anterior lobe becomes the seat of nutritional derangements that ought to be given time to run their course. In no case did the time interval between stalk section and thyroidectomy fall below three

weeks, which should have been ample time for necrotic cells within the local isthmus areas to be absorbed and vanish. As residues of such processes there arise cicatrices of connective tissue centrally in the lateral parts of the anterior lobe. In all cases the anterior lobe was well supplied with capillaries.

Whether it is the nerves or the portal vessels of the stalk which are of importance for the cell-reactions of the anterior lobe cannot be decided. Nor, probably, can this question be solved by experiments in which different centres of the hypothalamus are operatively destroyed. Certainly an important neurological question of localization might then be solved, but knowledge of the paths along which the impulses are transmitted will not be obtained by that means. An investigation by BORELL (1945) argues that the blood flow in the portal vessels passes in the direction from the pituitary, since he concludes that thyrotropic hormone from the hypophysis is deposited in tuber cinereum. Lastly, as a theoretical possibility unsupported by actual material, it may in this connexion be pointed out that intact portal vessels may conceivably be necessary for the pituitary to react to thyrotropic impulses of a nervous or other kind.

In the introduction it was mentioned that the significance of the stalk for pituitary function had been mainly analysed by indirect methods, *i.e.* experiments on the organs which are hormonally influenced by the pituitary. The experiments conducted here on the cell reactions in the pituitary represent a more direct procedure. By a similar method the cell-reactions were studied direct in the hypophyseal anterior lobe by WESTMAN and JACOBSON (1937). They found that stalk section prevents the arising of castration cells in the anterior pituitary of gonadectomized rats.

In cold-exposed rats with sectioned pituitary stalk BROLIN (1945) found that failure of the basophil cells to react to cold was coincidental with the failure of the increase otherwise arising in thyroid function to appear. Thus, there is full agreement between this result and those gained here under other experimental conditions.

The view that the thyrotropic function of the anterior pituitary is for the most part controlled without the mediation of the hypothalamo-hypophyseal pathway does not therefore seem to represent the actual conditions. It may further be added that the view in question is based on experiments in which the completeness of the stalk section has been open to doubt.

Summary.

Complete section of the pituitary stalk in the rat prevents hypertrophy and vacuolization from arising in the basophil cells of the anterior hypophyseal lobe after thyroidectomy. If the *pars tuberalis* connexion is left intact, however, these cell-changes do not fail to appear. When the posterior and middle lobes have been extirpated, typical cell-changes arise in the anterior lobe after thyroidectomy.

The changes in the basophil cell-picture are interpreted as a morphological sign of an increased thyrotropic function. Therefore, the thyrotropic function of the anterior pituitary lobe is regarded as highly dependent on the stalk connexion with the hypothalamus. The system composed of the hypothalamus, stalk and anterior lobe reacts to thyroid insufficiency independently of the intermediate and posterior lobes and the nerve fibres running through them. The view that the thyrotropic function of the anterior lobe is essentially controlled without the assistance of the stalk seems to be untenable.

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The Citric Acid Content of Older, especially Medieval and Praehistoric Bone Material.

By

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One hundred and fifty years ago the English chemist HATCHETT submitted his basic "Experiments and Observations on Shell and Bone". HATCHETT understood that bones from excavations might provide important evidence in the determination of the composition of the bone substance. His conception of the time necessary for the destruction or decomposition of a certain collection of bones was founded on experiments which he made on the os humeri of a man brought from Hythe, in Kent, and said to have been taken from a Saxon tomb.

My own interest in the citric acid content of bone material dates from DICKENS' discovery in 1940 of the high citric acid content of fresh bone material. The main problem was to determine the conditions for the extraction of citrate from the bone material. Contrary to what might have been expected from older solubility determinations the extraction of citrate was found to be a rather difficult process unless a strong acid was used.

There is another fact illustrating the difficulty of extracting citric acid from bone material. In these experiments it was thus observed that bones buried in the ground for long periods and exposed to the decomposing factors of the humus layers and the atmosphere, still showed varying amounts of citric acid.

The following observations were made by means of PUCHER, SHERMAN and VICKERY's method, with the improvements intro-

duced by HUNTER and LELOIR. All determinations were carried out on dried and finely pulverized material.

The investigations have been previously reported in a Swedish publication; the values obtained are given here in Table I, showing the citric acid content of certain medieval bone specimens.

Table 1.

Age		Ci-content in p.p.m.
Horse	uncertain	1,000
»	»	900
Cattle	16th century	2,200
»	»	3,400
Man	uncertain	1,800
»	»	1,300
»	1361 (the "Korsbetning" graves, Visby)	700
»	»	800
Horse	13th century	6,600
»	»	9,300
Man	uncertain (Ragnar Knaphövdes Chapel, Vreta Closter Church)	1,100

These figures can now be supplemented by analyses of considerably older material, reported in Table 2.

Table 2.

Locality	Approximate age of the find in years	Approximative Ci-content in p. p. m.
Lund	750	9,000
Stora Uppåkra, The Iron Age dwelling	1,500	1,600
Gualöv, Möllehusen	4,000	100
Ageröd, The Stone Age Dwelling	7,500—8,000	200

The age of the fragments could to a certain degree be determined under the microscope. The most recent fragments were least decomposed and the oldest most eroded. There are exceptions, however. Bone specimens dating from the same period may show rather different characteristics. A compact bone substance is evidently less easily affected by the destructive power of the humus layers and the atmosphere than is the spongy substance.

As an indicator of the age of the bone the Ci-content can be used only with the greatest reservation and under special conditions.

The examination shows thus that citric acid has been observed in all bones examined, even in such as are many thousand years

old. It is probable, however, that still older bones can be found which are entirely free from demonstrable citric acid content. If, however, the Ci-combination of the osseous substance is of cementlike character, the osseous substance may be decomposing into powder and lose its character of bone fragment simultaneous with the liberation of the citric acid component.

I wish to thank lecturer-curator H. BERLIN, professor E. SJÖVALL, Professor D. HOLMDAHL and the city veterinary I. SANDBERG, who have supplied the material and helped to ascertain its age. The technical analyses were carried out by miss ANNA-LISA SAGER and miss BIRGIT LUNDQVIST.

Summary.

Citric acid can be observed not only in fresh bone specimens (DICKENS) but also, in decreasing amounts, in bones which have been laying in the ground for thousands of years, exposed to the decomposing factors existing there.

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Studies on the Effect of Salyrgan on the Absorption of Water and Colloid from Joints.

By

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The mechanism of the action of mercurial diuretics is still a matter of discussion. The renal action of mercurial diuretics has been known for a long time (GOVAERTS 1928, HERRMANN and DECHERD 1937, GUKELBERGER 1944), and some authors state there is also an extrarenal action. Thus studies on alterations in blood chemistry of nephrectomized salyrgan treated rabbits have shown a decreased concentration of hemoglobin — interpreted as hydremia — and at the same time an increased concentration of the blood chloride (MÖLLER 1930). On individuals with intact kidneys, decreased values of hemoglobin and plasma proteins — interpreted as hydremia — have been found during the initial period of mercurial diuretic action and sometimes increased concentration of blood chloride has appeared. Both phenomena occurred before any great increase in the excretion of chloride and water by the kidneys appeared (SAXL and HEILIG 1923, BOHN 1924, CRAWFORD and MC INTOSH 1925, CLAUSSEN 1932, MÖLLER 1937). An extrarenal action of mercurial diuretics is also deduced from observations on the increased velocity by which intracutaneous saline wheals disappear on normal individuals (ENGEL and EPSTEIN 1931), as well as from observations of a more rapid flow of edema fluid from Curschmann needles placed in edematous tissue (HOFF 1925, TSCHERNING 1927, OFFENBACHER 1928) and a more rapid appearance in the blood of subcutaneously injected sodium fluoresceinate (DONATH and TANNE 1927).

However, this extrarenal action of mercurial diuretics is not generally accepted, but is denied by several investigators who have not been able to find any initial decrease in the concentration of plasma proteins (SCHMITZ 1933, BRYANS et al. 1935). Some authors working with this problem found a continuous decrease of the plasma volume (EVANS and GIBSON 1937, CALVIN et al. 1940). Others, on the contrary, found alterations in both directions (SWIGERT and FITZ 1940). The divergent results can possibly depend upon different experimental material.

In order to investigate the existence of an extrarenal action of mercurial diuretics, the authors have studied the absorption of water and colloid from the knee joint of rabbits before any increased diuresis has been caused by the renal action of the drug. The method used for the study of water and colloid absorption from the knee joints has been worked out by one of the authors (EDLUND, unpublished).

Methods.

Male rabbits were employed of about 2 kg of weight, supplied with usual food and water ad libitum. In the experimental series salyrgan treated and non-salyrgan treated rabbits have been used alternately with the same solution of hemoglobin.

Narcosis: 25 per cent urethane, 6 ml per kg of body weight, injected i. v. in order to get complete muscular relaxation. The injection of urethane lasted 10—15 minutes.

30 minutes after the end of the urethane injection blunt needles (length 18 mm, thickness 0.7 mm) were passed through the patellar tendon so that the point of the needle was located in the suprapatellar recess. The needles were then saturated to eliminate any tissue leakage. 0.85 ml, 5.5 g per cent, sterile, 38° C solution of human hemoglobin¹ made isotonic with rabbit plasma by means of sodium chloride and 10 mg per cent of calcium (CaCl₂), was injected as soon as the needles were in place. After 40 minutes 0.05 ml of the intraarticular fluid was withdrawn for determination of hemoglobin concentration ("the final concentration"). Immediately afterwards the joint cavity was washed out with 1.0 ml isotonic saline by means of ten consecutive injections and aspirations, and the concentration of hemoglobin in the washing

¹ The oncotic pressure of this solution, calculated according to ADAIR (1928) and corrected for temperature (38° C) according to WELLS et al. (1935), is 270 mm of water. The following formulas have been used:

$$\text{Oncotic pressure of the hemoglobin solution} = \frac{K}{V - B} = p \text{ (mm of H}_2\text{O)};$$

$$K = 3700; V = \frac{100}{\text{Hb conc. in g per cent}}; B = 2.54; p_1 = p \frac{t_1}{t}; t \text{ and } t_1:$$

absolute temperatures in degrees of C.

fluid was determined. From the values so obtained the absorbed amount of fluid and colloid was calculated.¹

The hemoglobin was determined as alkaline hematin according to WU's method (1922) modified by PETERS and v. SLYKE (1935). A Pulfrich photometer was used, filter S 50, 1 cm cuvette. The error of the method is ± 0.05 g per cent.

The mercurial diuretic used was salyrgan given in doses of 4–5 mg per kg of body weight. It was injected as a 1 per cent solution i. v. 10 minutes before the absorption period began in the right knee joint and 15 minutes before in the left knee joint.

The diuresis was measured on 6 animals during the first hour after the salyrgan was given by exposing the bladder and puncturing it. The volume of urine varied between 1.0–2.5 ml per hour, the same diuresis as in non-salyrgan-treated rabbits. As known from previous investigations one cannot expect a much increased diuresis in rabbits with the doses employed and the main diuresis appears later than one hour after the salyrgan injection (MÖLLER 1930).

The knee joints were kept in a flexion of 60° during the experiments. Animals who showed an amount of red blood corpuscles in the washing fluid exceeding 8,000 per cubic mm, and animals who moved their legs during the experiments were excluded. The body temperature was held within physiological limits during the experiments by means of an electrically heated operation table.

Results.

The absorption of hemoglobin and fluid was calculated for each knee joint and the means of the results from the two joints of each rabbit were treated statistically.

The following formulas have been used:

$$\text{Standard error of the mean} = \sqrt{\frac{S(X - \bar{X})^2}{n(n-1)}};$$

$$t = \frac{\bar{X}_1 - \bar{X}_2}{c\sigma_s} \cdot \sqrt{\frac{n_1 \cdot n_2}{n_1 + n_2}};$$

For the statistical meaning of "the t test", see YULE and KENDALL (1946).

¹ With the same method as previously described for the determination of the amount of hemoglobin absorbed, it has been shown in control experiments using suspensions of agglutinated red blood corpuscles instead of hemoglobin that the amount of blood corpuscles recovered equals the amount injected. In other control experiments similar to the absorption tests previously mentioned the joints have been washed with a concentrated suspension of red blood corpuscles (0.1 ml by volume), thus eliminating the intermittent increase in the intraarticular pressure, which accompanies the washing with saline. The results thus obtained show the same absorption values as when the washing was made with 1 cc of isotonic saline (EDLUND, unpublished).

The results are tabulated below.

Table 1.

	Untreated rabbits	Salyrgan treated rabbits	Differences \pm standard errors
	(n = 15)	(n = 14)	t values
The amount of hemoglobin absorbed in per cent of the amount injected	49.7 ± 2.4	59.0 ± 1.5	9.3 ± 2.8 $t = 3.28$
The absorption of fluid in ml	0.239 ± 0.027	0.419 ± 0.020	0.180 ± 0.034 $t = 5.18$
The final concentration of hemoglobin in gram per cent	3.86 ± 0.06	4.45 ± 0.07	0.59 ± 0.10 $t = 6.15$

From the table it is clear that *the absorption of colloid and particularly of fluid has increased during salyrgan treatment*. The final concentration of hemoglobin is higher among the salyrgan treated rabbits, indicating a relatively increased absorption of fluid. The statistical analysis shows that the differences are significant.

Discussion.

The results above show that the salyrgan treatment increases the absorption of water and colloid from the joints. This occurs before any increased diuresis can be measured as a sign of renal action of the drug. Joint cavities were used as convenient objects for studying absorption conditions of mesenchymal tissue, as they genetically can be looked upon as mesenchymal tissue spaces. For mesenchymal tissue it is stated that a colloid of a molecular weight matching that of hemoglobin is absorbed to the greatest extent (LEWIS 1921), or exclusively (FIELD and DRINKER 1931, BARNES and TRUETA 1941) via the lymphatics. Experiments made on absorption conditions of joints show no specific absorptive properties that make them differ from mesenchymal tissue (BAUER et al. 1933, RHINELANDER et al. 1939, ADKINS and DAVIS 1940, BAUER et al. 1940).

In the untreated animals the absorption of water from the joint cavity is possible both via blood capillaries and lymphatics. According to the current theories the passage of fluid from the capillaries and the absorption of fluid to them is regulated by the following factors:

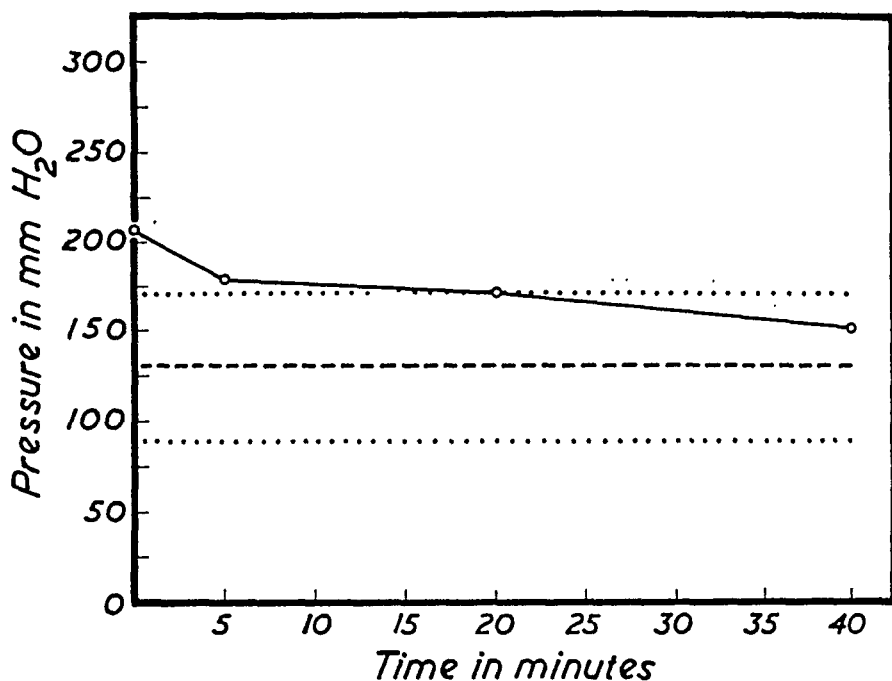


Fig. 1.

o———o the minimum pressure which will force water into the joint cavity (intraarticular oncotic pressure of the hemoglobin solution minus intraarticular hydrostatic pressure).

Each point of the graph is the mean of 15 independent values of oncotic (hemoglobin) and intraarticular hydrostatic pressures on a material which equals that of the untreated animals in this investigation. The oncotic pressure of the hemoglobin solution has been calculated according to the formulas previously referred to. The intraarticular pressure was measured with a compensation manometer. In no point of the graph the standard error of the mean exceeds ± 7 mm of water. (EDLUND, unpublished experiments.)

— · — · — · — · — average and range of calculated maximum pressure forcing water out of the joint cavity into the blood capillaries (oncotic pressure of the rabbit plasma 25.9–33.0 cm of water minus hydrostatic pressure in the venous end of the capillaries, 16–17 cm of water). See text.

1. Oncotic and osmotic pressure differences between the capillaries and the surrounding tissue.

2. Hydrostatic pressure differences between the capillaries and the surrounding tissue.

By regarding only the oncotic pressure of the hemoglobin solution and the intraarticular hydrostatic pressure during the 40 minutes the experiments lasted, the magnitude of the minimum pressure which will force water into the joint cavities (intraarticular oncotic pressure minus intraarticular hydrostatic pressure) is illustrated by figure 1.

The oncotic pressure of rabbit plasma is 25.9—33.0 cm of water (TADA and NAKAZAWA 1930). It is assumed that the capillary pressure in the venous capillaries is of the same magnitude in the rabbit as found in man, guinea pig and rat, *i. e.* 16—17 cm of water (LANDIS 1930 a, b). Therefore it is evident according to current theories, that one cannot expect any net absorption of fluid from the joint cavity to the blood capillaries, when one considers the magnitude of this minimum pressure which tends to force water into the joints. On the contrary, passage of fluid even from the venous end of the blood capillaries might be expected.

Neither can we presume any net absorption of fluid in the salyrgan treated rabbits, if only hydrostatic and oncotic factors are considered. The mean intraarticular oncotic pressure of hemoglobin is higher in these animals during the experiments and the intraarticular hydrostatic pressure presumably lower, owing to a decreased mean intraarticular fluid volume. Moreover the oncotic pressure of the plasma in salyrgan treated rabbits decreases during the first two hours after treatment, owing to a supposed hydremia (KYLIN 1932). A similar decrease in the oncotic pressure after salyrgan treatment is also observed in man (MEYER 1931).

The mechanism of this extrarenal action of salyrgan is unknown. Studies, already mentioned, on increased flow through Curschmann needles and earlier appearance of sodium fluoresceinate in the blood after treatment with mercurial diuretics (TSCHERNING, HOFF, OFFENBACHER, DONATH and TANNE) have been made on edematous individuals, a material so different from ours, that comparisons are useless.

The absorption of fluid in the way observed by us can be influenced by, among other things, alterations in the following factors:

1. *An increase of the total osmotic pressure of the plasma.* Such an increase is not improbable. MÖLLER (1930) observed an increase of the blood chloride values on nephrectomized rabbits, which he supposed to be caused by mobilization of tissue fluid, rich in chloride, to the blood. During the first hour after salyrgan treatment some investigators found increased values of plasma chloride on individuals with intact kidneys (SAXL and HEILIG 1923, BOHN 1924). Others, however, found normal chloride values and no great changes in the concentration of other ions (CLAUSSEN

1932, NOTHMANN 1933). Only small changes in the concentration of the blood electrolytes are required to cause important osmotic pressure differences (1 mN of chloride = 23 cm of water). Even if the effect of this osmotic pressure difference is determined by the differences in the diffusion velocities between water and electrolytes through the synovial membrane, and even if the effect lasts only for a short period, this factor cannot be neglected. The duration of the period during which intracutaneously injected saline wheals disappear with increased velocity after treatment with mercurial diuretics is only about an hour (ENGEL and EPSTEIN). This may imply that the increased absorption from the joints observed may be an effect found only during the first period of the drug action.

2. *Changes in the blood circulation of the synovial membrane.* A vasoconstriction is observed in the vessels of the rabbit kidney after salyrgan injections and in perfusion experiments on rabbit ears and frog hind limbs using concentrations of salyrgan within therapeutical ranges (MÖLLER 1932). A similar ischemia of the synovial membrane resulting in a decreased passage of fluid from the blood capillaries into the joint might give the impression of an increased absorption of fluid.

3. *Increased absorption by the lymphatics.* Earlier published investigations on the effect of mercurial diuretics on the flow of lymph from the thoracic duct (MELVILLE and STEHLE 1928, CLAUSSEN 1932, WATKINS and FULTON 1938) do not permit any conclusions about the lymphatic absorption from the knee joint.

If any of the factors mentioned cause the increased absorption of water observed by us, it is at present impossible to decide. Changes in the permeability of the endothelium of blood and lymph capillaries or changes in the permeability of connective tissue similar to those caused by the action of "spreading factors" might be of importance, but no such effects of mercurial diuretics have been found in the literature.

The increased absorption of colloid may be caused by the higher mean concentration of hemoglobin, following the increased absorption of water in the salyrgan treated rabbits. Similar results concerning the absorption of hemoglobin have been found in untreated animals, if the mean concentration of colloid is higher during the experiments (EDLUND unpublished).

Summary.

The authors have found an increased absorption of colloid and particularly of water from the knee joints of normal rabbits treated with salyrgan during the first hour after the administration of the drug. Some possible explanations of this action of the drug are discussed, but the mechanism remains unapprehended.

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An Instrument for Producing Brain Lesions in the Rat.

By

NILS-ÅKE HILLARP.

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The instrument which has almost exclusively been used previously is HORSLEY and CLARKE's (1908) stereotaxic instrument. There has never been any important modification of the original construction of this although the technique for localisation of the brain lesion has recently been improved by combining X-ray photography (RANSTRÖM, 1947). For large laboratory animals, especially the cat, this instrument works completely satisfactorily, but its complicated and relatively coarse adjustment mechanism renders it less suitable for rats. The need which often arises now for small precise bilateral symmetrical lesions, exactly localised, thus also necessitates the construction of another instrument, easily manipulated, working with adequate precision, and based on another principle.

Description of Instrument.

Holder for the head: Fixation of the head in a definite position in relation to the electrode is done by means of an appliance which is removable from the remainder of the instrument (Figs. 1 and 2). The metal tongue *c* is placed in the animal's mouth so that its rubber cushion presses on the upper molars. The tips of the incisors rest in the groove *b* and are held in position by the strap *a* which screws firmly over the nose. By means of the screw *f* the head can be moved in a rostro-caudal direction, whilst the screw *d* gives a suitable inclination in the sagittal plane. The spiral spring of the screw *e* presses the head up against

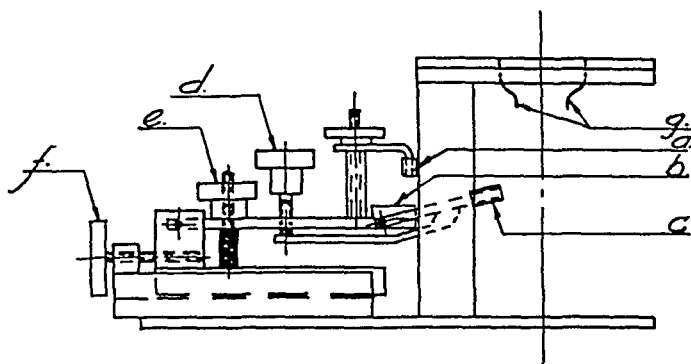


Fig. 1. Cross section through the holder for the head. 2/3 actual size.

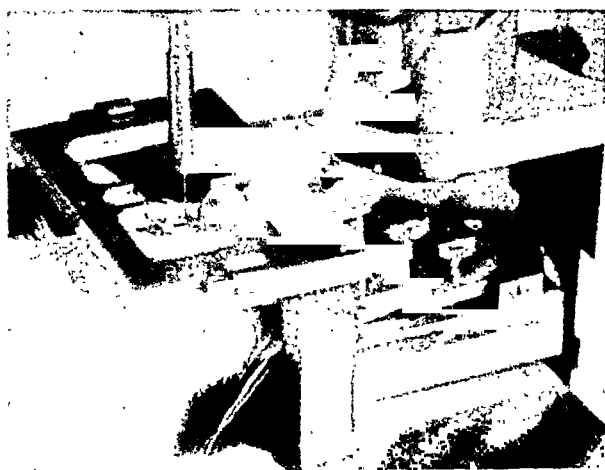


Fig. 2. Detail of the head holder showing application of fixation pins.

the three fixation pins *g* in such a way that the single rostral pin comes to be placed in the intersection between the coronal and sagittal sutures. The caudal pair of pins together with the previous pin fix the roof of the skull perpendicularly to the electrode. — The dimensions of the holder are suited to female rats of weight 170 g. to 210 g.

Electrode guide: The construction of this is seen from Figs. 3 and 4. The electrode holder runs in a cylinder of bakelite material to prevent lateral movements. It is suspended by a support from a shaft, the rotation of which raises or lowers the electrode and which is mounted in such a way that the electrode may be held at any desired height. The weight of the electrode holder is arranged so that it just causes the electrode to sink down

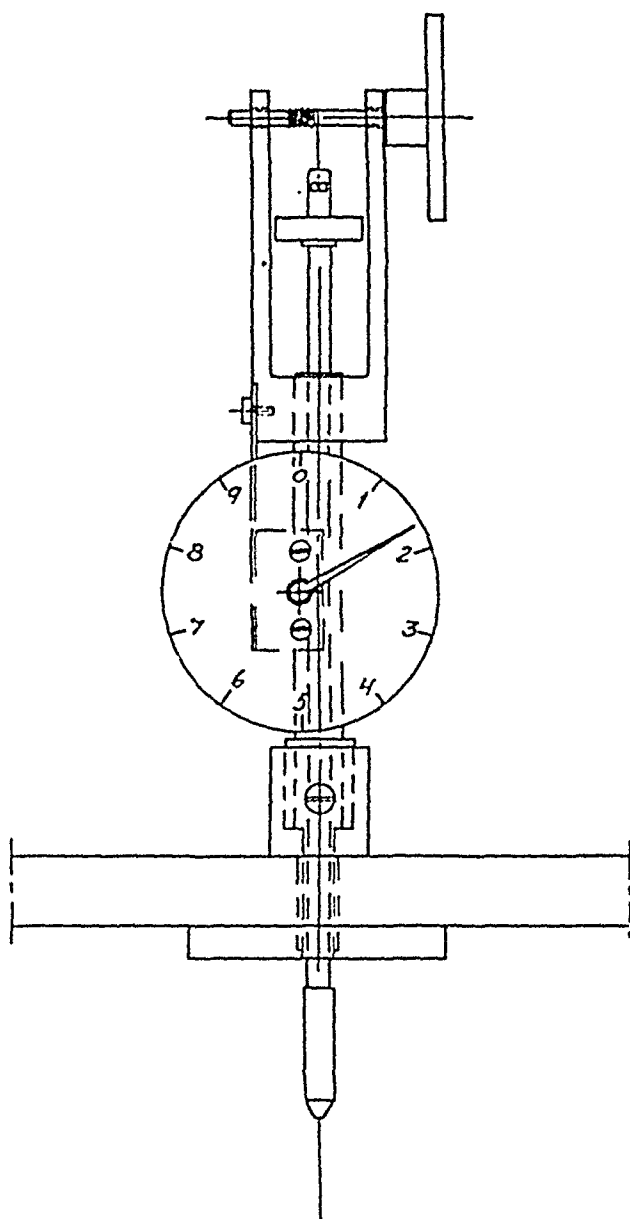


Fig. 3. Electrode guide seen from the front. 2/3 actual size.

through the brain but not through the basal dura. The height of the tip of the electrode above the base of the skull or its depth under the cortical surface can be read with an accuracy of 0.05 mm on the indicator scale (see Fig. 3). The electrode may suitably consist of a nichrome wire of 0.3 mm diameter insulated

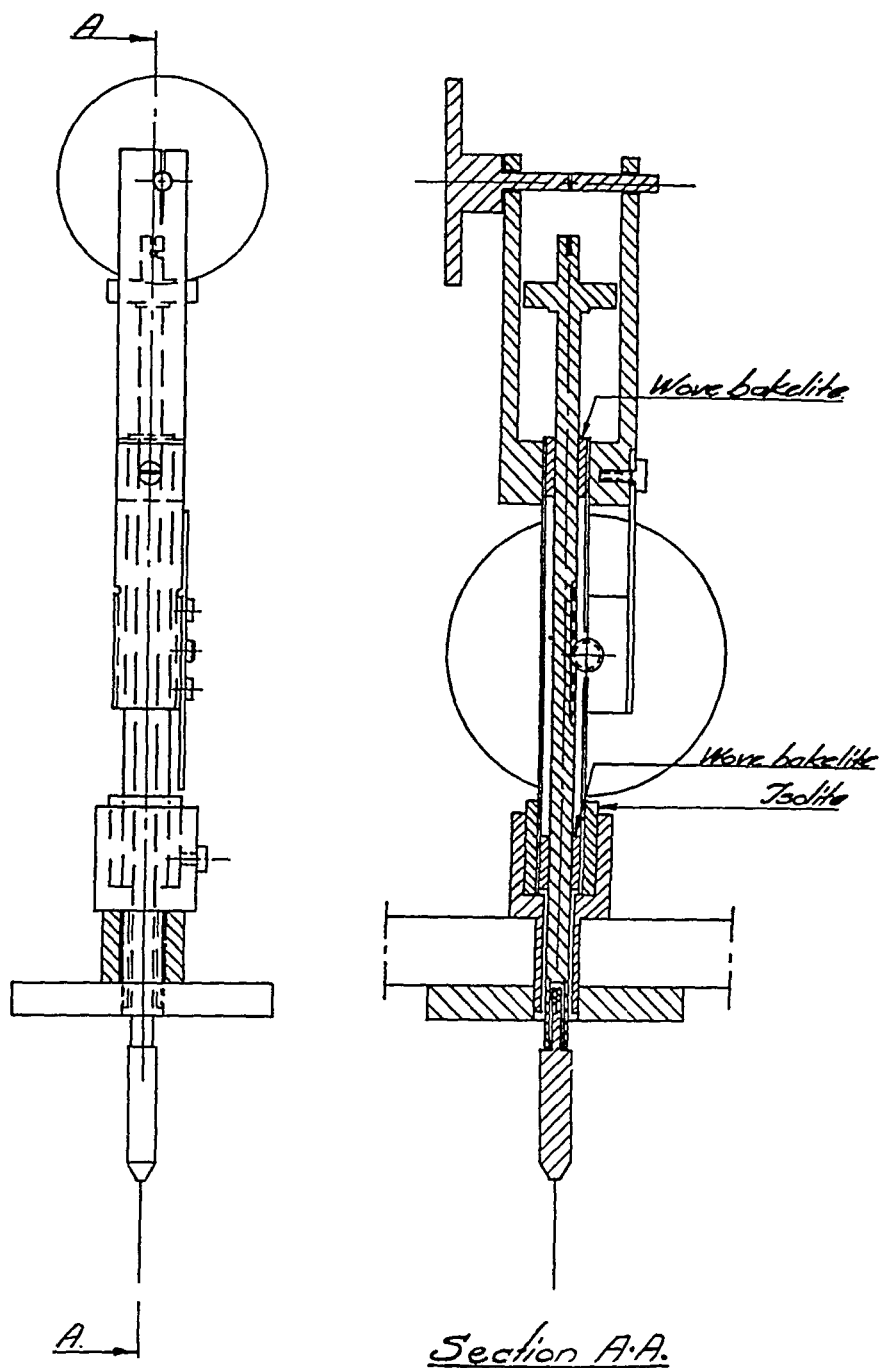


Fig. 4. Cross section through the electrode guide. 2/3 actual size.

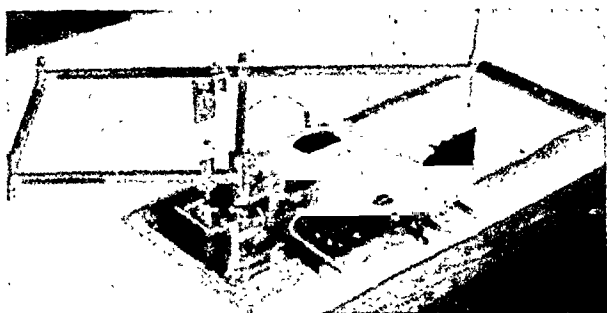


Fig. 5. General view of the entire instrument with the movable pantograph pencil and its co-ordinate system on the right.

with shellac except for the blunt tip which is free from insulation for a distance of 0.3 mm.

Arrangement for the adjustment of the electrode in the horizontal plane: The adjustment is based on the pantograph principle as will be seen from Fig. 5. The construction is such that the electrode moves one tenth of the distance traversed by the pantograph pencil. This pencil can be moved within a system of co-ordinates drawn on millimeter cross-ruled paper. The zero point of this co-ordinate system is that position of the pencil where the electrode is situated immediately above the tip of the rostral fixation pin. By moving the pantograph pencil the tip of the electrode is brought to bilaterally symmetrical points to within 0.1 mm.

Electrical circuit: The electrode is connected as an anode to a circuit containing a 12 volt battery through a fixed resistance of 1 k Ω (to prevent a short circuit if the resistance between anode and cathode should approach zero), a variable resistance of 50 k Ω and a milliammeter. The metal tongue c (Fig. 1) serves as a neutral electrode.

Lesion Technique.

Under nembutal anaesthesia (4.2 mg intra-peritoneally per 100 g body weight) a mid-line incision is made through the skin over the roof of the skull. Two bilaterally symmetrical holes are then drilled through the bone down to the dura at the desired site. The head is then fixed in its holder and the tip of the electrode brought to the desired region of the brain by means of the pantograph co-ordinate system and height adjustment of the



Fig. 6. Frontal section through the hypothalamus of the rat. Bilateral symmetrical lesions in the rostral part of n. supraopticus. Gallocyanin-chrome alum according to EINARSON. 10 μ section. $\times 17$.

electrode holder. (Just as in the HORSLEY-CLARKE stereotaxic instrument the co-ordinates, for example those of a required brain nucleus, must, of course, first be determined empirically by a serial section examination.) For basal lesions the most reliable height adjustment is attained if the electrode is first permitted to sink to the base of the skull by its own weight and is then raised to the required height. The electrolytic lesions are produced by a current of 0.5 to 1.0 mA applied for 20 to 60 seconds depending on the size of the lesion required.

A necessary condition for reproducible results is, of course, that the variations in the position of the intersection of the coronal and sagittal sutures are not large. It has been found that this condition is fulfilled if inbred (8th generation) rats of the same sex and weight are used. Fig. 6 shows the bilateral symmetry for lesions in the rostral part of n. supraopticus.

Summary.

An instrument based on the pantograph principle for producing bilateral symmetrical brain lesions in rats is described.

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Studies on the Biochemistry of Human Semen.

II. Some Properties of Prostatic Phosphatase.

By

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Investigations on the enzymology of the "acid" phosphatase present in human semen and prostatic tissue have been carried out mainly by KUTSCHER, the discoverer of this enzyme and his coworkers (KUTSCHER and WOLBERGS (1935), K. and WÖRNER (1936), and K. and PANY (1938)). They have established a considerable irreversible inhibition by various organic solvents with narcotic properties such as alcohols, urethane and even ethyl ether. A solution of the enzyme treated with ether for one hour is completely inactivated. The authors assume that this phenomenon is connected with the high state of purity of their preparations. Against this view speaks the observation that differentiation between prostatic and other acid phosphatases in human sera may be obtained on treatment with alcohol (HERBERT, 1944). The prostatic phosphatase is strongly inhibited by fluoride, as is the rule for acid phosphatases. This inhibition is due to some kind of competition between fluoride and substrate for the enzyme (KUTSCHER and WÜST, 1941). The findings of OHLMEYER (1942) that magnesium is present in prostatic phosphatase as an activator and that the fluoride inhibition is increased on addition of magnesium seem to suggest a rather complex interrelation involving possibly a Mg-enzyme-fluoride respectively Mg-enzyme-substrate complex.

The optimum pH for the prostatic enzyme was found by KUTSCHER and WÖRNER (1936 II) to be 5.2—6.2, when glycerophosphate was used, and, 4.0—5.4 when phenyl phosphate was used as a substrate. These determinations were, however, only approximate. STRÖJER RASMUSSEN (unpublished data) in the case of phenyl phosphate found optimum pH to be 5.8—6.2, whereas FROM HANSEN (1946) and RIISFELDT (1946) found pH 5 to be the optimal reaction for the same process. GUTMAN and GUTMAN (1940) investigated the acid phosphatase in serum from patients with metastasizing carcinoma of the prostate and found the optimum pH to be about 5. Thus there appears to be some divergence on this point.

The purpose of the present investigation is to throw some light on the splitting of phosphoryl choline, the natural substrate of the prostatic phosphatase (LUNDQUIST, 1946, 1947) as compared with the phosphoric esters commonly applied in phosphatase experiments, β -glycerophosphate and phenyl phosphate and thus to find out whether the enzyme is especially well adapted to the conditions prevailing in human ejaculate.

Optimum Conditions for the Enzyme Activity.

In order to compare the activity of prostatic phosphatase towards the three phosphoric esters mentioned, it was essential first to determine the optimal hydrogen ion concentration for the process. Attempts were made to determine the pH-activity curves at constant ionic strength as this factor is often neglected, though it may sometimes be of considerable significance. Buffer solutions containing oxalic ($pK_2 = 4.3$), fumaric ($pK_2 = 4.5$), succinic ($pK_2 = 5.5$) and maleic acid ($pK_2 = 6.6$) were made in such a way that the ionic strength was the same throughout the interval pH 3—pH 7.5. This was facilitated by using a relatively small concentration of buffer in 0.1 m sodium chloride. A platinum electrode in the buffer solution containing a mixture of ferro- and ferricyanide showed a nearly constant potential throughout the pH interval used. The electrode potential is independent of pH, but highly sensitive towards variations in ionic strength (BRODERSEN, 1944). When the activity was tested in these buffers it was, however, observed that the curves obtained were not smooth but exhibited considerable displacements when shifting from one buffer substance to another. This phenomenon was most evident with oxalate buffer which in the concentration used ($2.5 \cdot 10^{-2}$ m) brought about a strong inhibition of the enzyme. Inhibition of phosphatases by oxalate has been observed by other authors.

The procedure finally adopted for determining the pH-activity relation was to use acetate buffers of constant ionic strength and

to take advantage of the buffer properties of the substrate substance itself in the region pH 5.5—7.5. The solutions were prepared in the following way:

Acetate buffers were made by mixing 0.15 m sodium acetate with 0.15 m acetic acid containing 0.15 m sodium chloride. Any mixture of these solutions have the ionic strength 0.15. The substrate solutions were made by mixing a 0.05 m solution of the secondary sodium salt with a solution of the primary salt, obtained from the secondary solution on addition of the calculated amount of hydrochloric acid plus 0.05 m sodium chloride. Through this procedure the ionic strength is kept constant at 0.15. The solutions used in the experiments are mixtures of equal parts of buffer and substrate of approximately the same pH. The pH has been controlled in all cases by potentiometric measurement with a glass electrode using a Radiometer instrument. The substrate concentration in the final solution is 0.025 m and the ionic strength 0.15. The same substrate concentration has been used throughout without regard to different affinity between the enzyme and the three phosphoric esters employed. In the case of phosphoryl choline, the calcium salt was used, though this means a deviation from the principle of constant ionic strength. The phosphate liberated was determined by the method of FISKE and SUBBAROW (1925) using a FISHER electrophotometer. The enzyme preparations were either prostatic secretion or seminal fluid, diluted approximately 500 times with water and centrifugated.

Typical curves are reproduced in figures 1—3. The net result was that the optimum reaction for the splitting of glycerophosphate and phenyl phosphate is very nearly the same, viz. pH = 6. The pH optimum of phosphoryl choline splitting appears to be a little to the alkaline side of 6 (about 6.3), under the conditions described. In 0.1 m citrate buffer it was found to be 5.2. No special precautions were taken with regard to the ionic strength in this case.

The inhibition of prostatic phosphatase by various substances was investigated in some detail. It was observed both with anionic and cationic substances: oxalate, maleinate, fluoride and zinc. It was further noticed that the inhibition in some cases was diminished or prevented by citrate. When 0.02 m citrate was added to the buffer-substrate mixtures described above a rise in phosphate liberation was ascertained in the case of β -glycerophosphate (generally from 10 to 30 % but sometimes more) and phosphoryl choline — both the calcium and sodium salts — (from 100 to 500 %), but not or only to an insignificant degree with phenyl phosphate. This finding was quite surprising in so far as citrate has been found rather to inhibit this phosphatase some-

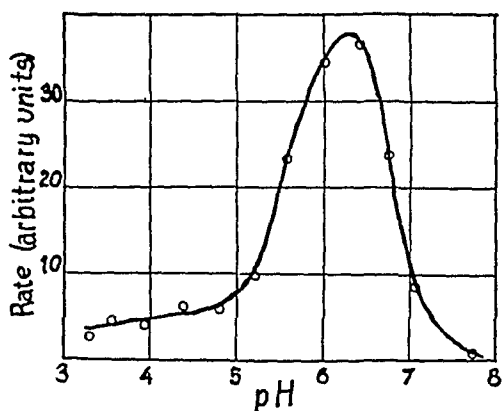


Fig. 1. The relation between pH and the rate of hydrolysis of Calcium phosphoryl choline chloride in acetate buffer. Substrate concentration 0.025 molar.

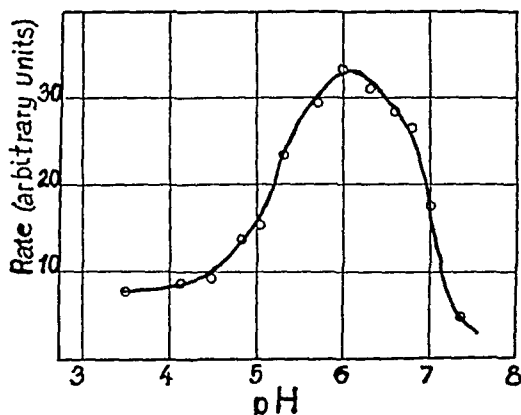


Fig. 2. The relation between pH and the rate of hydrolysis of Sodium β -glycerophosphate in acetate buffer. Substrate concentration 0.025 molar. Ionic strength 0.15.

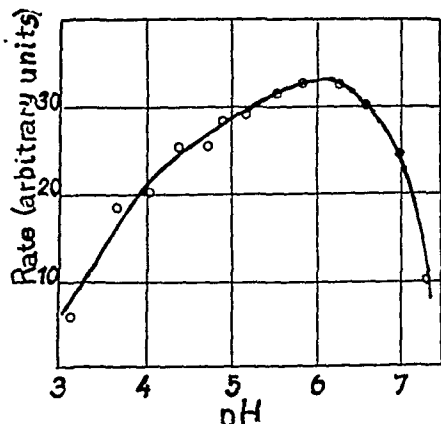


Fig. 3. The relation between pH and the rate of hydrolysis of Sodium phenyl phosphate in acetate buffer. Substrate concentration 0.025 molar. Ionic strength 0.15.

what (GUTMAN and GUTMAN (1940)). The possibility of impurities in the phosphoric esters employed was considered. Two preparations of sodium phenyl phosphate were used; one synthesised according to FREEMAN and COLVER (1938) in this laboratory, the other was a pure product from the British Drug Houses. Two preparations of sodium β -glycerophosphate were also used, one synthesised by the writer, the other from B. D. H. Calcium phosphoryl choline chloride was synthesised by the method of PLIMMER and BURCH (1937). It was a beautiful white crystalline substance showing the composition reported by PLIMMER and BURCH. In the first preparations technical calcium hydroxide

was used for neutralising the phosphoric acid excess. It was a possibility that this substance was contaminated with fluoride. A batch made with calcium hydroxide prepared from the purest calcium chloride behaved, however, similarly. In addition to the activating or anti-inhibitory effect of citrate it was also observed that the double determinations agreed much better when citrate was added, most striking in the case of phosphoryl choline.

Table 1.

Inhibition of prostatic phosphatase with various substances and the influence of citrate on the inhibition. Substrate 2.10^{-2} m β -glycerophosphate, pH 6.0. (Acetate buffer.) The results are expressed as % of the value without addition of inhibitor, ("normal"). The normal values with citrate addition were 115—120 % of those without citrate.

Addition	Conc. mol/liter	Rate of phosphate liberation, % of normal	
		without citrate	with citrate, $1.67 \cdot 10^{-2}$ m
normal	—	100	100
Sodium fluoride	$1.67 \cdot 10^{-3}$	0	43
—	$8.4 \cdot 10^{-4}$	0	111
—	$1.67 \cdot 10^{-4}$	100	98
Zinc chloride	$2.0 \cdot 10^{-2}$	6	—
—	$1.67 \cdot 10^{-3}$	75	100
Sodium oxalate	$1.67 \cdot 10^{-2}$	55	55
Sodium maleinate.....	$1.67 \cdot 10^{-2}$	53	99

Table 1 shows some typical inhibition experiments. Under the circumstances of the experiments, oxalate is the only one of the four substances investigated which is not counteracted by citrate. BAMANN and SALZER (1936) have observed a similar anti-inhibitory effect of citrate with Taka phosphatase. They observed that the phosphatase preparations contained an inhibitor which was counteracted by citrate (0.04 m). This anti-inhibition was not seen below pH 4, a fact which may perhaps indicate that the enzyme is more susceptible to inhibition at low pH values. If this is the case with prostatic phosphatase the difference in shape of the pH-activity curves of phenyl phosphate on one side and glycerophosphate and phosphoryl choline on the other

may be due to the greater affinity of phenyl phosphate (see below) for the enzyme and the resulting small susceptibility to inhibition by some naturally occurring inhibitor. If the inhibition be abolished all three curves probably should be very much like the phenyl phosphate curve. BAMANN and SALZER further observed that citrate addition displaced the pH optimum towards the acid region. A similar phenomenon may perhaps occur in the case of prostatic phosphatase thus accounting for the discrepancy between the results of the present writer and those of the majority of other investigators, and also explain the result with phosphoryl choline in citrate buffer, mentioned above.

The activation through fluoride appearing in table 1 at intermediate concentrations when citrate is present has been confirmed on repetitions of the experiment. Any influence of citrate on the inorganic phosphate determination (compare LUNDSTEEN (1938)) was not observed at the low concentrations used. Phosphate standards containing the different substances have been used throughout, but no influence on the colorimeter value was observed. In the following experiments on affinity and activation energy addition of 0.02 m citrate was made the rule in order to eliminate the influence of small amounts of inhibiting substances, evidently often present in the enzyme preparations.

The Energy of Activation.

Phosphoryl choline is a rather remarkable substance being a strong base as well as a strong acid. The substance crystallises at the same time as chloride and calcium salt and in solution it must be present as an amfoion within most of the pH scale. It was considered of interest to investigate the activation energy when this compound is split by prostatic phosphatase as compared with the activation energy of glycerophosphate and phenyl phosphate splitting. The activation energy for the hydrolysis of β -glycerophosphate by bone phosphatase from cat and man was found by BODANSKY (1939) to be 9,940 cal/mol in both cases. The present experiments were made with 0.020 m substrate in acetate buffer as described above. The citrate concentration was 0.02 m. Four different temperatures were used: 0, 12.5, 25, and 37 degrees centigrade. The time of reaction was adjusted after preliminary experiments in such a way that the same degree of hydrolysis was obtained at all temperatures. Fig. 4 shows the

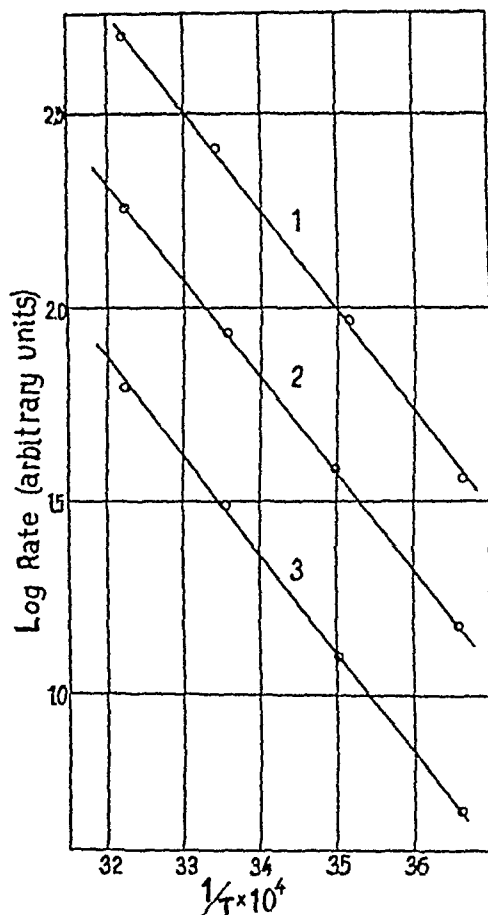


Fig. 4. Evaluation of activation energies. Substrate concentration 0.020 molar. Citrate concentration 0.020 molar. Acetate buffer, pH = 6. Curve 1. phenyl-phosphate, curve 2. β -glycerophosphate, curve 3, phosphoryl choline. The relative position of the curves in vertical direction is quite arbitrary.

results when log of the reaction rate (in arbitrary units) is plotted against the inverse of the absolute temperature. The slope of the straight lines times the gas constant R measures the activation energy, A .

$$A = RT^2 \frac{d \ln v}{dT}$$

The numerical values found were:

β -Glycerophosphate	11,300 cal/mol
Phenyl phosphate	11,800 —
Phosphoryl choline	11,700 —

The difference between these values is probably within the experimental error. A survey of the scant literature on the subject (cf. *e. g.* SIZER (1943)) makes it clear that it is not a general rule that an enzyme shows the same activation energy towards different substrates. Any interpretation of the fact will not be attempted here.

The Affinity between Enzyme and Substrate.

Experiments were made to estimate the dissociation constants of the enzyme-substrate complexes (the Michaelis constant). KUTSCHER and WÖRNER (1936) have measured the reaction rate at various concentrations of β -glycerophosphate. These results may be used for determining the dissociation constant in question. When m/v is plotted against v , m being the substrate molarity and v the velocity of phosphate liberation, a straight line should be obtained if the reaction follows the simple scheme of Michaelis and Menten (cf. LINEWEAVER and BURK, 1934). The slope of this line equals the velocity at infinite substrate concentration (V), and the intersection with the ordinate axis is K_m/V . The results of KUTSCHER and WÖRNER (1936) arrange themselves about a line giving K_m about 0.028 in the one case and 0.015 in the other case (with "gealtertes Ferment").

The experimental solutions employed in the present experiments were prepared by mixing the substrate and buffer solutions of pH 6.0 in various proportions. At the highest concentration used — 0.04 m — no buffer was added, the buffer capacity of the substrate itself being sufficient. All the solutions had equal ionic strength. A large number of these experiments with Ca phosphoryl choline chloride were unsatisfactory and had to be discarded. This was probably due to the influence of the calcium ions on the phosphate determinations. When the sodium salt was used, very good results were obtained. A solution of the sodium salt was prepared from the calcium salt on addition of sodium carbonate, oxalate being avoided as a means for precipitating the calcium ion, on account of the inhibition mentioned above.

Table 2 shows the results of these experiments. The dissociation constant (Michaelis constant) and the affinity between substrate and enzyme, — ΔG are recorded. ΔG is calculated as $RT \ln K_m$. When citrate is added and the substrate concentration is 0.025 m, phenyl phosphate is hydrolysed about twice as rapidly as the two other substances.

Table 2.

Affinity between prostatic phosphatase and various substrates. K_m is the Michaelis konstant as obtained from curves like those of figures 5—7. The concentration of citrate, when added, was 0.02 m.

Phosphoryl choline	Temp.	Citrate	K_m^{-1}	$-\Delta G$ (cal/mol)	ΔH (cal./mol)
Calcium salt	37°	—	62.5	2,500	
—	37°	—	62.7	2,540	
—	37°	+	97.4	2,810	
Sodium salt	37°	+	46.7	2,360	
—	0°	+	45.8	2,150	(0)
β -Glycerophosphate					
Sodium salt	37°	+	54.0	2,450	
—	37°	—	78.7	2,680	
—	37°	+	95.4	2,800	
—	0°	+	129	2,640	(1,600)
Phenyl phosphate					
Sodium salt	37°	—	1,130	4,300	
—	37°	—	942	4,210	
—	37°	+	1,065	4,280	
—	0°	+	1,350	4,070	(1,100)
—	0°	+	1,290	4,040	(1,300)

A few experiments were made at 0° in order to investigate the dependence of the affinity on temperature, thus making possible an evaluation of the heat of reaction and the entropy change in the binding process. We have:

$$\Delta H = RT^2 \frac{d \ln K_m}{dT}$$

When ΔH and ΔG are known ΔS may easily be obtained from: $\Delta G = \Delta H - T \cdot \Delta S$. Some typical experiments are shown in figures 5—7 in order to give an impression of the experimental error, which is in fact considerable, when it is taken into account that the uncertainty in evaluating the small distance on the ordinate axis is determinative of the error in the evaluation of K_m . The difference found between K_m at 0° and 37° is probably within the experimental error. We may state that $\Delta H = 0 \pm 1,500$ cal/mol for the three reactions considered. In the case of phosphoryl choline and glycerophosphate ΔS is calculated to about

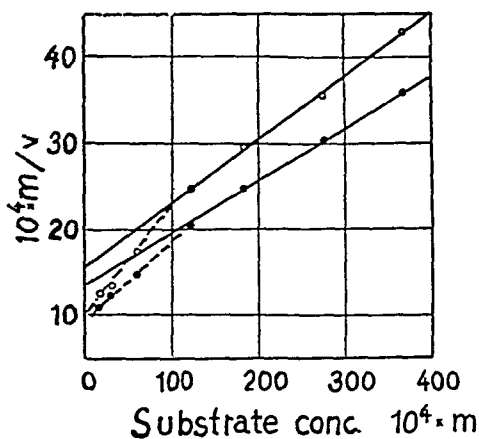


Fig. 5. Evaluation of the binding between substrate and enzyme. Sodium phosphoryl choline chloride in acetate buffer, pH = 6, O at 37°, ● at 0° Celsius.

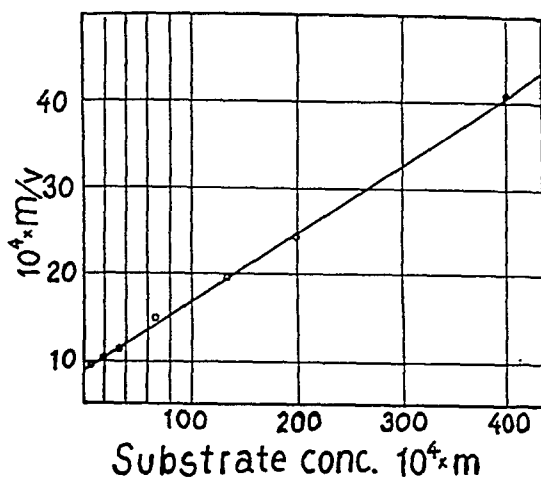


Fig. 6. Evaluation of the binding between substrate and enzyme. Sodium β -glycerophosphate in acetate buffer, pH = 6. Temperature 37° C.

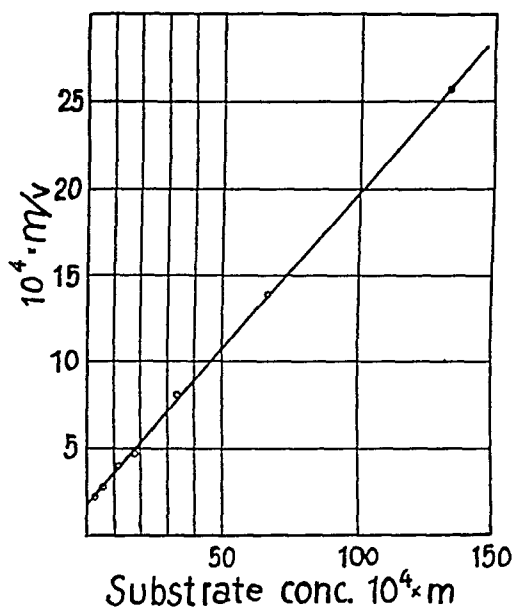


Fig. 7. Evaluation of the binding between substrate and enzyme. Sodium phenyl phosphate in acetate buffer, pH = 6. Temperature 37° Celsius.

8 cal/degree and mol and for phenyl phosphate to about 12 cal/degree and mol. Similar results were obtained by VEIBEL and ERIKSEN (1936, 1937) for the enzymatic hydrolysis of various β -glucosides.

Discussion.

The human prostatic phosphatase is apparently rather well adapted for the hydrolysis of phosphoryl choline. The optimum activity is reached at a pH, which is possibly encountered in semen after contact with the acid vaginal mucus. Now these measurements are concerned with a very dilute enzyme solution and the possibility must be faced that the pH optimum is not the same under natural conditions, *i. e.* high concentration of enzyme, proteins and other substances. The hydrolysis of phosphoryl choline in human ejaculate was, however, found to be very rapid (LUNDQUIST, 1947).

The affinity between enzyme and substrate is approximately the same for phosphoryl choline and β -glycerophosphate as is also the rate of hydrolysis in the presence of citrate. The investigations of SCHERSTÉN (1936) have shown that large amounts (about 0.5 per cent) of citrate are in fact present in human semen. This similarity in the splitting of glycerophosphate and phosphoryl choline means that the presence of the positively charged quaternary Nitrogen does not affect the interaction between substrate and enzyme.

If the equilibrium constant for the enzymatic hydrolysis of phosphoryl choline could be measured at two different temperatures, a complete thermodynamic treatment of the enzymic process might be obtained, ΔG and ΔH for the first stage of the process: the combination of enzyme and substrate, being known. Measurements of the enzyme catalysed equilibrium between glycerophosphate and glycerol + inorganic phosphate have been performed by KAY (1928), giving a value for $-\Delta G$ of about 2,300 cal/mol (cf. LIPMANN (1941)). This result shows that the change in thermodynamic potential (free energy) is exclusively due to the combination between enzyme and substrate. In the case of phenyl phosphate the corresponding value of ΔG is not known, and is not likely to be obtained from enzymatic experiments, but it appears to the writer quite possible that the aromatic phosphate esters might have a higher group potential (LIPMANN, *l. c.*) than the aliphatic ones so that the high value found for the affinity between phenyl phosphate and phosphatase might also correspond to the total change in thermodynamic potential on hydrolysis; but this assumption is purely hypothetical.

I wish to acknowledge the considerable interest which Prof. KNUD SAND, chief of the institute, has taken in my work. I am much indebted to Miss RUTH FAHRNER for her very careful technical assistance.

Summary.

The enzymatic hydrolysis of phosphoryl choline, the natural substrate of human prostatic phosphatase, β -glycerophosphate and phenyl phosphate has been studied.

1) The optimum pH when measured in acetate buffer solutions of constant ionic strength, is found to be about 6.0 for glycerophosphate and phenyl phosphate, and about 6.3 for phosphoryl choline.

2) Fluoride, zinc chloride, oxalate and maleinate are found to inhibit the enzyme to a varying degree. Citrate to some extent abolishes this inhibition, and furthermore increases the reaction velocity of glycerophosphate and, especially, phosphoryl choline hydrolysis, also when prostatic secretion or seminal plasma is used without added inhibitor.

3) The energy of activation is found to be about 11,500 cal for each of the three processes.

4) The affinities between enzyme and substrate were measured at 37° and 0°. For phosphoryl choline and glycerophosphate it is about 2,500 cal/mol (37°), and for phenyl phosphate about 4,200 cal/mol. The change in heat function is small ($\Delta H = 0 \pm 1,500$ cal/mol).

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the Vitamin Institute, Stockholms Högskola.

Nucleic Acids and Cytological Changes in the Thyroid Gland after Thiouracil.¹

By

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The chemical inhibition of the thyroïdal function by thiouracil, producing a total hypothyrosis of the organism (McKENZIE and McKENZIE 15, ASTWOOD, SULLIVAN, BISSEL and TYSLOWITZ 2) is an interesting problem in cytophysiological respect. By the influence of thiouracil (according to ASTWOOD (1) more generally by the influence of substances characterised by the groups $\text{NH}\cdot\text{CS}\cdot\text{NH}$ —, or $\text{NH}_2\text{C}_6\text{H}_4$ —) the thyroid shows symptoms, which are strikingly similar to a state of high activity, but it does not produce any hormon. The inhibition of the production of the hormon was recently proved by a method with labelled iodine by RAWSON, TANNHEIMER and PEACOCK (24), KESTON, GOLD-SMITH, GORDON and CHARIPPER (16). The follicular cell, however, differs thereby by its morphological signs diametrically from the state in the resting gland (either naturally inactive, or after potassium iodide).

CHAIKOFF and co-workers, having placed by experiments with labelled iodine the synthesis of di-iodotyrosine and thyroxine into the follicular cell, determined by the same way, that thiouracil blocks this synthesis. It could be assumed, that the chemical inhibition of the glandular function is undoubtedly the work of a disturbance in the synthesis of thyroxine. Against this two

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objections can be made, referring partly to the process of iodation of tyrosine, partly to the character of the thyroïdal hormon in general.

As to the biosynthesis of thyroxine MANN and co-workers (21) declare, differently from the predecessor, that di-iodotyrosine can probably result also from outside the gland. Also CHAPMAN (8) counts on the base of his experiments with the extrathyroidal origin. The blockade of the synthesis of thyroxine in the follicular cell could not produce the hypothyrosis of the organism by itself, if the substance is naturally produced outside the gland as well. However, as thiouracil, according to the morphologic symptoms, affects the follicular cell and stops the secretion of the hormon, it must probably affect the cellular function more deeply than in the section of the iodation of tyrosine only. Up to a certain point this is shown by the observation, that *e. g.* it disturbs the activity of the system tyrosine-tyrosinase (DEMPSEY 9).

The mentioned conception of the deeper influence of thiouracil on the chemical processes of the cell touches on the problem of the character of the thyroid hormon. The above mentioned explanation of CHAIKOFF deals only with thyroxine. The more recent endocrinological studies show however more and more clearly that this substance is not the only and sole specific product of the thyroid (McCLENDON and FOSTER 7, KENDALL 13), and they pay their attention to thyroglobuline, the proteinic character of which has been subjected to new researches (LUNDGREN 19, 20). Contrary to the fact that under normal conditions thyroglobuline can never be found in the peripheral blood, it was found here with the aid of a sensitive serological test during abnormal function of the gland (LERMAN 18, STELLAR and OLKEN 28). Under physiological conditions the thyroglobulinic molecule splits probably into smaller proteins of the size of albumine and these are transported into the blood-circulation; the splitting of thyroglobuline can be attributed to the proteases present in the colloid of the thyroid gland (DE ROBERTIS 25). If therefore the thyroglobuline also shows a deeper coherence with the action of the thyroid, thiouracil which inhibits the function of the gland, would have to disturbe the process of its formation as well. This is also in line with the formerly formulated conception about the deeper influence of the inhibitor on the action of the follicular cell.

By a more detailed histophysiological study of the inhibited glands we came to the conclusion that thiouracil affects the cells

of the thyroid more seriously, than in the section of the iodation of tyrosine only. By comparing the microscopic symptoms, characterising the normal function (KROGH and OKKELS 17, UOTILA 30, later PONSE and ALTSCHULER 23), substantial deviations in the structure of follicles and in the morphology of cells are evident. After a long lasting action of thiouracil the follicles show signs of multiple enlargement, but they are empty and collapsed; only a prolonged fissure is visible, which is a remainder of the former lumen. The gland has a compact appearance, owing not only to the compression of the follicular cavities but also to the increase of the substance of the tissue. The cells have outspoken cylindrical form, they are often extremely prolonged and thin like sticks. Their cytoplasm shows a loss of affinity against acid plasmatic dyestuffs and it has a tendency to absorb slowly haematoxyline. On the other hand the caryoplasma stains itself less abundantly than normally. The comparison with the morphological signs of the physiological phases of function of the gland shows generally the following differences: the form of the follicular cavities differs from the inactive gland as well as from the secreting one. The form of the cells reminds of hormonal secretion, it is however more strikingly cylindrical. The affinity for stains of the cytoplasm is atypical. According to all these signs thiouracil does not get the glandular cell into the state of inactivity; the follicular cell is active, but as if running empty, without result, without production of a specific increte.

A very striking phenomenon of the activity of thiouracil, which we were finding regularly in the inhibited glands is an abundant occurrence of mitotically dividing cells (ŘEŘÁBEK 26). Mitoses are a rare phenomenon in a normal thyroid gland; their number in connection with physiological secretion according to UOTILA (*l. c.*) does not increase considerably. Some authors (BASTÉNIÉ and ZILBERZAC 3) observed the increase of the number of mitotically dividing cells, the difference however was not more than double. PONSE and ALTSCHULER (*l. c.*) are of the same opinion, that the secretion of the hormon happens without any proliferation. The striking frequency of the mitotic division after thiouracil seemed according to him to be a direct manifestation of the activity of this substance.

The influence on the intensity of the mitotic division is due generally to the quantitative changes of the desoxyribonucleic acid of the nucleus, but the glandular activity in general as well

— at least as far as to the glands producing proteinic secretions — shows a connection with the metabolism of nucleotides (CASPERSON 4, 5, 6). Some histophysiological observations, made with the aid of coloured microreactions (Feulgen's, orcine) directly in the tissue, seem to testify, that the nucleotides are probably in close connection especially with the processes of the physiological function of the thyroidal cell. Recapitulating the statements of the literature up to now, it is shown that during the production of the colloid the nucleotidal reactions in the follicular content are intensive, whereas in the nuclei they grow weak. During the proper secretion of the hormon the cells form a secret rich in nucleotides, whereas in the nuclei their concentration shows a decrease. It was stated during the production of the basophilic colloid that the nuclei are emitting drops into the cytoplasm which give clear nucleic reaction, and the resulting basophilia of the ripe colloid is attributed just to those emissions, passing finally over to the follicular cavities (UOTILA *l. c.*).

If therefore the nucleotides seem to be firmly connected with the secretory actions of the thyroidal cell, it is still more interesting, that these actions are inhibited just by thiouracil, by a substance so near to one of the bases of ribonucleic and desoxyribonucleic acid. This fact, in connection with our above mentioned preliminary observations about the cytomorphology of the thiouracil action, leads to the question of its encroachment into the metabolism of nucleoproteins and their building components, nucleic acids. It is especially the raised frequency of mitotic division which points towards this direction. Starting from this point, we subjected firstly the influence of thiouracil on the mitotic division of follicular cells to a more detailed research from the cytological point of view, and in connection herewith we made quantitative-analytical investigations about the occurrence of both nucleic acids of the cytoplasm and the nucleus.

Experimental.

Experiments were performed on albinotic rats, which were fed with thyroidal inhibitor (we used 4-methyl-thiouracil as it is more intensively active) in daily doses 50 mg per os by an oesophageal sound. The cytological investigations were made on preparations, stained especially with Azan (azo-carmin—aniline-blue—orange) besides the current haematoxyline technique. All preparations were tested by Feulgen's reaction. The quantitative statistics of mitotic division

were made in connection with the rising number of the doses up to the total number of ten, in paraffine sections of thyroids using Feulgen's reaction. From the series of sections, in which every gland was separated, samples were selected, coming partly from the edge parts of the gland, partly from the center. In each of these preparations 17,000 nuclei on the average were examined and from these the number of those was stated which were found in the stage of mitosis. Besides the statistics of the mitoses measurements were also made in sections of the size of the follicular cells and cell nuclei. The measurements were made directly under the microscope using an immersion objective and a micrometric eyepiece. Breadth, highness and thickness of the cells were measured, the volume of the cell was counted as the volume of a hexaedron. Both axes of the nuclei were always measured, the average value taken and the volume was counted as the volume of a ball. The number for the quantity of cytoplasm was gained by subtraction of the volume of the nucleus from the volume of the cell as a whole.

For the extraction and for the determination of ribonucleic acid (RNA) served the method of v. EULER and HAHN (25); desoxyribonucleic acid was determined according to DISCHE (26).¹ Owing to the small weight the thyroids of two rats were always extracted and the original prescription was modified into a micromethod, in which the volume of the gross extract was always 4 ccm, precipitated with 0.5 cc of 2 % lanthan acetate and the sodium salts of nucleic acids were dissolved again in the total volume of 4 cc. About the coefficient of the analytic exactity of v. EULER's and HAHN's reaction and the reaction of DISCHE the same can be said as in the determination of nucleic acids in other organs (ŘEŘÁBEK 27).

Results.

1) Normal Rats Thyroids.

The glands of normal animals, coming from the breeding, showed the normal picture characteristic for the rat's thyroid, *i. e.* the state passing from inactivity over to a mild hormonal secretion. The follicles were of medium size, approximatively circular or slightly elliptical, filled with a viscose colloid, basophilic in the center. The vacuolisation in the peripheral zones of the colloid showed some slight secretion. The epithelium of the follicles had cells mostly of the shape of pavement stones, chromophilic, with small, intensively staining nuclei. Cubical follicles were in the minority and only occasionally there was the cylindrical type with basically situated nuclei, *i. e.* secretory epithelium. The

¹ As the extraction method used gives exact results only for RNA, the data given below for DNA are somewhat too low (comp. EULER a. HAHN, Arkiv f. Kemi 25 A, No. 11 (1947)).



Fig. 1. Normal Thyroid gland of the Rat. Haematoxyline-cosin. 450 \times .



Fig. 2. Thyroid gland after 2 doses of Thiouracil. Haematoxyline-cosin. 450 \times .

secretion of the control animals was only a weak one within physiological limits. Fig. 1 shows an example of one of the control glands.

According to the microscopical measurements the average volume of the thyroidal cell of the used normal rats was $569.5 \mu^3$, out of which the volume of the nucleus was $66.6 \mu^3$ and the corresponding volume of cytoplasm $502.9 \mu^3$.

The mitoses in control animals were a rare phenomenon, which could be estimated with difficulty by exact statistics. The figures in single animals were only fractions of one pro mille and showed considerable individual deviations. The normal frequency of mitoses in rat's thyroid can therefore be numerically stated only with difficulty, not regarding the fact, that such a figure is very variable in connection with different breeding races and cultivation conditions. The average value in the used animals, representing the arithmetical average of the single measurings, was 0.4 ‰ .

Every set of analyses of nucleic acids showed almost completely identical values, differing only slightly. The partial statements are shown in table 1. The average content of ribonucleic acid (RNA), counted on 1 g of fresh weight of the thyroid, was 9.21 mg, the content of desoxyribonucleic acid (DNA) 8.34 mg. From this follows the relation RNA/DNA 1.1. The weight of rat's thyroids showed to be very constant and amounted averagely to 16.5 mg.

Table 1.

	RNA			DNA		
	mg per 1.0 g of gland		%	mg per 1.0 g of gland		%
	Results	Average		Results	Average	
	9.20 9.18 9.21 9.23	9.21	100.0	8.23 8.44 8.28 8.40	8.34	100.0
Normal						
After 3 doses of Thiouracil	8.60 8.50	8.55	92.8	7.44 7.40	7.42	89.0
After 10 doses of Thiouracil	5.36 5.45 5.40 5.30	5.38	58.4	4.75 4.00 3.72 4.48	4.24	50.8
After 60 Units of Thyrotropic horm. of the anter. Pituitary lobe	8.29 8.33	8.31	90.3	9.19 9.21	9.20	110.3

2) Thyroid after Initial Doses of Thiouracil.

The observations in this group include the period up to 4 daily doses of thiouracil. The follicular cells showed in this time microscopically a constant increase of volume; after 2 doses they were cubical (Fig. 2), after 4 they became cylindrical. The cytoplasm showed from the beginning an atypical colouring and it had a cloudy structure. The interior of the follicles was reduced to a mere fraction of its normal size; after 2 doses it contained still some rests of chromophobe filling, after 4 doses it was completely free of colloid. The nuclei were hypertrophic, slightly staining, granular or in form of a network and they seemed to show Feulgen's reaction always more and more weakly. Their localisation inside the cell was at first not uniform, but later basal in accordance. The total picture of the gland differed distinctly both from state of inactivity and from secretive action.

The frequency of mitoses showed a constant rising tendency. Their number surpassed after 2 doses already the value of 4 ‰, after 4 doses it was more than 5 ‰ of the total number of follicular cells. Microscopically the dividing spindle could be hardly found and the grouping of chromosomes corresponded in general to the metaphase (Fig. 2, 4). The result gave the impression of



Fig. 3. Thyroid gland after 4 doses of Thiouracil. Haematoxylin-eosin. 450 \times .

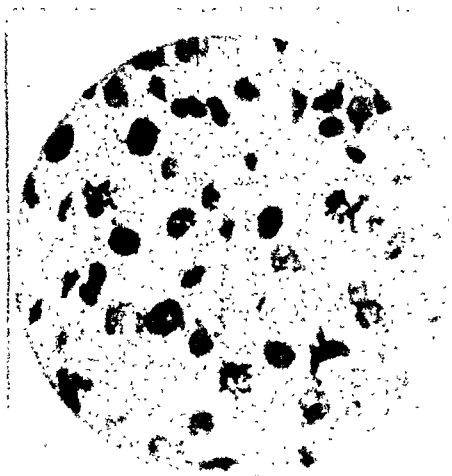


Fig. 4. Feulgen's reaction in a Thyroid gland after 4 doses of Thiouracil. 450 \times .

a disturbance of the mitotic process in this section. Also after 4 doses the configuration of the chromosomes in the dividing cells corresponded to metaphase, but in comparison with the above mentioned it was considerably damaged. A deformation of chromosomes and the occurrence of short stick or ball forms was often stated (Fig. 3).

At the periphery of the gland we remarked during the period of 4 doses signs of desquamation of the cells. This is a phenomenon, which was observed by some authors in glands with a highly active secretion, but it was considered as an artefact in consequence of the spongy consistence of the cells (PONSE and ALTSCHULER *l. c.*). We also reckoned with this possibility and payed a special attention to the working in divided groups; the result — to which we shall return later — was however unchanged.

The microscopical measurements, however, showed that after 3 doses, when the cells had already a cylindrical form, the volume was of the average of $1,715 \mu^3$, *i. e.* it was increased to thrice the normal state. The nucleus showed the same growth, it reached $199 \mu^3$. The volume of the proper cytoplasm had $1,516 \mu^3$, which is again three times the original normal size.

The quantity of nucleic acids showed at that time (*i. e.* after 3 doses) a decrease. In 1 g of gland tissue 8.55 mg of RNA and 7.42 mg of DNA were found on the average. The relation RNA/-

DNA was 1.2 and it was therefore only slightly increased in comparison with the normal.

Table 2.

	Size of cells		Size of nuclei		Volume of cytoplasm in μ^3
	in μ	Volume in μ^3	Radius in μ	Volume in μ^3	
Normal Thyroid gland of the rat	8.6×7.7	569.5	2.5	66.6	502.9
After 3 doses of Thiouracil	11.4×13.2	1,715.5	3.6	199.3	1,516.2
After 10 doses of Thiouracil	9.2×18.4	1,557.4	3.2	140.0	1,417.4

4) Thyroid after 4—10 Doses of Thiouracil.

In proportion with the growing number of doses the cells obtained a more and more marked cylindrical form, until they

were after 10 doses extremely protracted into form of sticks (Fig. 5). The nuclei were relatively smaller, a little bit more chromophile than before and they were localised characteristically basally. The cytoplasm showed a granular structure and chromophobic vacuols were present, which were especially striking after 10 doses (Fig. 5), when they reminded the phenomenon of resorption of the colloid, although the shrunk follicles did not contain any secretion



Fig. 5. Thyroid gland after 10 doses of Thiouracil. Haematoxyline-eosin. 450 \times .

and kept only the cellular detritus. It came from the cells, decomposing in growing measure. The decomposition attained after 6 doses whole coherent parties of epithelium on the periphery of the gland and sometimes touched also the central district. After 8 doses the extent of desquamation began to diminish and after 10 doses it seemed to be completely stopped.

The frequency of mitoses began to sink slowly after 6 doses; on the average 3.3 ‰ of the cells were found in caryokinesis, after 8 doses the average sank again to 2.7 ‰ and finally after 10 doses it reached the value of 1.4 ‰. The frequency of mitoses during the whole observing period formed a curve, represented in Fig. 6. In the final phase the mitotic figures obtained the normal form, the deformation of chromosomes decreased.



Fig. 6. Frequency of mitoses in glands. Vertically: number of mitoses p. 1,000 cells. Horizontally: doses of Thiouracil.

During the period of 10 doses the follicular cell had the average volume of $1,557.4 \mu^3$, *i. e.* only slightly smaller than after 3 doses, but always approximately three times the normal volume. The nucleus, with its volume of $140 \mu^3$ was between the double and triple of the normal size, similarly was also the volume of cytoplasm ($1,417 \mu^3$).

It is interesting that also the total weight of rat's thyroid was after 10 doses of thiouracil approximately the triple (40.2 mg) of the normal one.

The quantity of nucleic acids in the weight unit of the gland had during the dosage of thiouracil a constant sinking tendency and it reached the minimum (RNA 5.38 mg/g, DNA 4.24 mg/g), after 10 doses. The relation RNA/DNA rose above normal and also above the value found after 3 doses of thiouracil (1, 3).

If the dosage of thiouracil continued further, the result did not change against that mentioned before. The dividing cells occurred in a quantity of a fraction of one pro mille, the follicles consisted of high cylindrical cells with basally placed nuclei and they were free of secretion. The signs of cell's destruction disappeared.

We observed the glands in some cases up to 40 days having fed with regular doses of thiouracil and we found a constant unchanged state.

Discussion.

The summary of original morphological results show that the cells of the follicular epithelium receive during the dosage of thiouracil constantly more and more expressive signs of form, which exceed largely those by which the cell, secreting a hormon, is characterised, but which are also entirely different to the morphological characteristics of the inactive follicular cell. The influence of thiouracil proves microscopically that, even if the thyroïdal cell does not produce any hormon, it does not show a resting state. It has some symptoms reminding of those of secretory activity, but it differs in details expressively from the cell producing the hormon and from the cell assimilating the colloid. Thiouracil therefore does not bring the follicular cell in a state identical with physiological inactivity as *e. g.* potassium iodide. The stoppage of the production of the hormon seems therefore to be the result of a non-physiological damage of the gland's function.

The mentioned changes are accompanied by a temporary rise of the number of mitoses in the epithelium. This action does not lead to the rise of double-layered epithelium or of double-nucleic cells; the mitoses show an abnormity in form and configuration of the chromosomes, their course is inhibited in the stage of metaphase. The rising of the number of the mitotically dividing cells seems therefore not to be the result of a formatively irritating impulse. If we judge this phenomenon from the point of view of the statements, according to which the secretion of the thyroïdal hormon is not connected with the proliferation of glandular cells (UOTILA *l. c.*, PONSE and ALTSCHULER *l. c.*), the appearance of mitoses after thiouracil represents a manifestation of the toxic activity of the substance on all the vital actions of the cells. If we consider the fact, that according to BASTÉNIÉ and ZILBERZAC (*l. c.*) the physiological secretive activation of the gland is accompanied by a rise of mitotic division to approximately the double of the normal, the reaction to thiouracil is basically similar to the symptom of secretion, but by its intensity it exceeds this accompanying symptom grossly. An interesting analogy of that is also the described result of the destruction of epithelium

in the peripheral zones of the gland. This phenomenon was stated by a number of authors in the phase of hormonal secretion, but it is lately specified as an artefact (PONSE and ALTSCHULER). We observed it after thiouracil regularly to a considerable extent and we did not succeed in removing it neither with the most careful working of the material. It seems therefore, that similarly as in the case of morphological changes of the follicular cell, it is here a symptom of pseudosecretion which exceeds by its extent the physiological state and signalises again the toxical influence on the vitality of the cell. This is a demonstration which results summarily from all microscopical observations, corresponding to the fact, that by the action of thiouracil the thyroid gland is not brought into the stage of physiological inactivity, but derailed in a pathological state.

If we ask for the reason of this action, an interesting contribution is given by research of the nucleic acids of the gland. The absolute quantity of ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) according to the analyses carried out, decreases together with the dosage of thiouracil. To obtain a comparable scale, we analysed also thyroids secretively activated by the thyrotropic hormon of the anterior lobe of the pituitary gland, of which every animal got in total 60 unities in partial daily doses. After the dosage of hormon the glands had all microscopic symptoms of hyperthyreosis. The analysis of nucleic acids showed, that whilst the quantity of RNA per gram of tissue slightly decreased, the quantity of DNA rose considerably (see tab. 1). The latter rising can be considered as significant of DNA for the secretion cycle of the thyroid gland and we shall deal with it in a separate study. If we compare the result in the gland inhibited by thiouracil with the result in glands activated by the hormon of the pituitary gland, the extent of the damage, caused by the inhibitor, is shown.

We were now interested to see how the relative concentration of nucleic acids in the follicular cell manifests itself in connection with the found absolute sinking of their quantity in one weight unit. During the transition of the gland into the pseudosecretion type the colloid disappears and consequently it must be reckoned with a certain decrease of nucleic acid contained in it. This quantity, which is unknown, causes a certain fault in the valuation, but this covers only the interval of the first 3 days of the dosage of thiouracil, because after this time all colloid is restlessly driven out. Simultaneously the cells grow, but as they don't grow in

quantity, the gland is formed by a quantity of cells roughly comparable to those at the beginning. If the glandular tissue contains after 3 days of thiouracil dosage a reduced quantity of RNA and DNA (tab. 1) and a threefold increased substance of cytoplasm and nucleus (tab. 2), it shows that the relative concentration of both original nucleotides in the cell sank to less than the third part of the original concentration. Even if we attribute the absolute decrease, stated analytically after 3 days' dosage to the loss of colloid, the relative decrease of the concentration of the nucleotides in the cell is a third of the normal one. By continuing the dosage the content of RNA and DNA in the gland sinks constantly (tab. 1) by an unchanged increase of the mass of the cell (tab. 2). In the final phase of ten days dosage the relative concentration of RNA in the cytoplasm corresponds to less than 20 % of the normal concentration, and of DNA in the nucleus.

Consequently by the action of thiouracil the content of nucleotides in the cell sinks, the absolute quantity as well as especially — with regard to the increased substance of the cell — the relative concentration in the cytoplasm and nucleus. This is a very interesting phenomenon, caused by a substance, which represents as a matter of fact one of the bases of ribonucleic acid (uracil), respectively deoxyribonucleic acid (methyluracil). A certain analogy for this phenomenon can be found in the observations of v. EULER (30), who stated that the dosage of yeast, RNA and DNA reduces the index of nucleotides in the liver cells of rats. v. EULER looks for the reason of this decrease in the oxidoreductive processes of the liver cell and he found a concordance herewith in the analogical reaction of the tumor tissue where he found earlier the inhibition of enzymatic processes.

For the time being it cannot safely be decided, whether the action of thiouracil on the nucleotides of the thyroid, observed by us, can be taken as a specific influence on the thyroidal cells or only as a partial activity of its influence on the cells in general. But our observation that the number of cells in mitosis in the liver tissue of animals, which had thiouracil hypothyreosis, is not increased and no morphologic changes can be stated, proves against the latter conception. We are studying the metabolism of the nucleotides under the influence of thiouracil in other tissue cells in a further special study.

But whether thiouracil attains the nucleotidal metabolism of

the thyroïdal cell specifically or only in connection with the analogical reaction in the cells of other tissues, its action in the thyroïdal cell according to the observations made is unusually effective and for the thyroid gland of a functional significance. We have already mentioned before all the connections of the secretory action of the follicular cell with the metabolism of nucleotides. Also the characteristic reaction, stated by us in the process of mitosis seems to be a further testimony; the violent decrease of DNA stated by chemical analysis corresponds well with the disturbance of caryokinesis. Based on all these circumstances we believe to see a serious reason for the inhibitive influence of this substance on the thyroïdal secretion in the depressive action of thiouracil on the nucleotides of the follicular cell.

Summary.

1) By the action of thiouracil the follicular cell is not brought into the state of physiological inactivity and it shows signs of apparent secretion which differ however in details remarkably from the effective secretion stage.

2) The quantity of follicular cells in mitosis is temporarily increased to the multiple. This disturbance is the result of the inhibition of caryokinesis in the stage of metaphase.

3) The absolute quantities of ribonucleic and of desoxyribonucleic acid are considerably reduced.

4) The ratio RNA/DNA shows a decrease to a fractional part of the normal value, according to the increase of the cell mass.

The authors thank here the head of both Institutes in which the work was performed, Prof. H. v. EULER, for the amiable leaving of all necessary resources.

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Obituary Notice.

JOHANNES LINDHARD.

1870—1947.

Professor J. LINDHARD, who died on the 11th of October 1947, had a somewhat unusual scientific career. Born on April 25th 1870 in the little Danish town of Dalby (Seland), he went into the army shortly after having finished school. But this work did not satisfy him, so he took up studies again and in 1893 entered the University of Copenhagen, where he passed his medical examination five years later. After spending some time as a physician at the Ivigtut mines in Greenland, he practised for a few years in Denmark. Being greatly attracted by research work he decided, however, to test whether he would be suited for such tasks. The realisation of this plan was made possible when he took part as a physician in the Denmark expedition to the northeast-coast of Greenland during 1906—1908. From that time his decision was taken, and he now devoted himself entirely to physiology, at first at the Finsen Institute, where he collaborated with HASSELBALCH, and later with KROGH at the zoophysiological laboratory. LINDHARD soon found a wide and fruitful field of his

own in the study of bodily exercise in its different aspects. A lectureship in anatomy and the theory of gymnastics was created for him in Copenhagen in 1909, and in 1917 he became professor in the theory of gymnastics. Somewhat later he got a small laboratory of his own, and when the new Institute of Physiology was erected in Copenhagen in 1928, one of its five departments was devoted to the theory of gymnastics with LINDHARD as its first chief. LINDHARD also acted for several years as Director of the State Institute of Physical Education. After his retirement in 1940 he continued to work regularly in the laboratory, where he was still active a week before his death.

From LINDHARD's extensive scientific production only a very brief summary of the main trends can be outlined here.

During his stay on Greenland, LINDHARD measured several bodily functions such as skin, mouth and rectal temperatures, the red and white cell counts, carbon dioxide output, ventilation etc. A special interest attaches to the observation of seasonal variations in respiration, with low respiratory rate but maximum alveolar ventilation and minimum alveolar carbon dioxide tension during summer. LINDHARD obtained similar results in Copenhagen and with HASSELBACH (1911) found that the ultraviolet radiation increased the excitability of the respiratory centre. This was determined through the reaction to carbon dioxide according to the principle introduced by LOEWY, but the method was now considerably improved and subsequently further developed by LINDHARD (1933). The study with KROGH with the aid of KROGH's bicycle ergometer and respiration apparatus proved that already the first respiration at the onset of muscular work became increased in rate and depth, which must be due to cerebral impulses (1913). For electrically induced work a reflex from the working muscles started the initial increase (1917). In both cases a chemical regulation of respiration set in later. An important paper by LINDHARD on statical work (1920) demonstrated that in this case the greatest increase in ventilation as well as in gas exchange might be found after the work, the damming up of the blood flow in the working muscles leading to an anaerobic breakdown with increased acidity of the blood after release of the flow. The accumulation of acid products explains the fatigue during work of this type. Valuable observations were also made on the composition of the alveolar air and the respiratory dead space in different conditions.

In order to follow the corresponding alterations in circulation it seemed necessary to measure the general blood flow. A method for determining the minute volume of the heart by means of the absorption through the lungs of an indifferent gas, as suggested by BORNSTEIN, was worked out by KROGH and LINDHARD (1912), using nitrous oxide for this purpose. This has been a very useful tool in numerous investigations, until it was superseded by the acetylene and the so called direct methods. LINDHARD found that during rest the minute volume of the heart is a function of the respiratory metabolism (1916), though physical factors like the body position and ultraviolet irradiation (1913) also influence it. Above all he studied the effect of muscular work: when the gas exchange rose from the resting value in proportion to the amount of work performed until a maximum about ten times as great was reached, the minute volume of the heart showed a parallel though somewhat slower increase up to a maximum of six times the resting value. This signifies that the utilization of the oxygen of arterial blood is increased during muscular work (1915). Training enabled the subject to perform the work with relatively smaller cardiac output per minute — but a greater output per beat — than the untrained subject.

In an extensive investigation KROGH and LINDHARD took up the question concerning the substances which are metabolised during muscular work (1920). It turned out that at a respiratory quotient during rest of 0.8 to 0.9 only a slight change took place during work, whereas with high quotients during rest, a lowering, and with low resting quotients, a rise in the working period was observed. It was concluded that during work the same substances are burnt as during rest and in the same proportions. In the special conditions with low or high quotients, conversion of fats to carbohydrates or the reverse was to be assumed. The experiments also proved that when fat is catabolised for muscular work a waste of 11 % of the heat of combustion of the fat takes place. Later on LINDHARD came back to the subject (1927), since it had been maintained that muscular work of short duration takes place exclusively on carbohydrates, and gave convincing evidence that this is not the case.

A number of papers deal with the functions of the muscles themselves. In most of them LINDHARD collaborated with others, mainly with younger colleagues who were inspired by his enthusiasm.

After HENRIQUES and LINDHARD (1920, 1923) had obtained evidence that the action potentials of the skeletal muscles are intimately associated with processes in the motor end-plates, it seemed possible that the physiological stimulation of the muscle fibre is of electrical nature and generated in the end-plates. This led to determinations with BUCHTHAL (1934, 1935), which showed that during rest the potential gradient from end-plate to muscle fibre, which could be abolished by curare, is much greater than in the fibre itself. The study of the structural changes during isometric contractions (1936, 1940) showed that the anisotropic layers were shortened whereas the isotropic parts became extended. It was also found that the elasticity coefficient of the muscle decreased during the isometric contraction — similar results being obtained at the same time by WEBER and by STEINHAUSEN.

By means of needleshaped thermocouples the temperature in muscles were measured by BUCHTHAL, HÖNCKE and LINDHARD (1944). Although the resting muscle of the extremities was usually found to have a temperature some degrees below the rectal temperature, in the working muscle a rise was observed which was directly dependent on the magnitude of the work. In static work the rise continued after cessation of the work.

Several other papers by LINDHARD should be mentioned, *e. g.* his study on the influence of different gymnastic body positions on the thorax, his methods for the determination of the hydrogen ions in small quantities of blood, his work on dye-methods for determination of the blood-volume and so on. Above all his numerous textbooks on the theory of gymnastics must be referred to. Here he criticized severely many current views on physical education, its aim and means; he showed that in numerous instances such views had been founded, not on a solid scientific basis, but on faith and were to a great extent the result of propaganda. But he himself more than anybody else also gave positive contributions to our understanding of this vast and important field.

LINDHARD had a very independent judgment which he expressed freely and with great ease. Sometimes this gave to his writings a more polemic touch than was necessary. In the personal contacts he was at first a little shy, at least when meeting colleagues outside Scandinavia. This was undoubtedly due in part to his lack of training in speaking foreign languages — he sometimes complained that he had not had the opportunity of going abroad

in his youth. For those who had the privilege of knowing him intimately he became a most reliable friend, interesting and well-informed. His life work, the result of clear thinking and unusual energy, will give him a permanent place among those who have laid the foundations to our knowledge of the physiology of muscular exercise.

G. Liljestränd.

The Influence of Some Central Nervous Depressants on the Reciprocal Inhibition Between the Two Retinae as Manifested in Retinal Rivalry.¹

By

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The reciprocal inhibition between the two retinae when they are presented with different pictures, a phenomenon well-known as retinal rivalry, bears obvious similarities to the inhibition or so-called suppression in cases of strabismus.² With the possibility of therapeutical application to such cases in mind, we have investigated the influence of some drugs on retinal rivalry. There seems to be no reference to similar experiments in previous literature.

Methods.

1. Principle of the Method Used.

The objects used in the present study are illustrated in fig. 1 which is intended to be observed through a standard stereoscope. The circles with the horizontal and vertical bar were situated on illuminated squares at the center of large black fields. The fields are not shown but only indicated by the black border around each square. The contours of the squares and the circles are intended to be fused so that rivalry is confined to the crossed bars.

¹ A preliminary communication was made at the meeting of the Swedish Ophthalmological Society Nov. 23rd, 1946. (Nord. Med. 34, 1448, 1947.)

² We have previously shown that during retinal rivalry there is an inhibition of the pupillary light reflex from the momentarily suppressed eye. (BÁRÁNY and HALLDÉN 1948.) This fact has been interpreted as possibly indicating the existence of phasic inhibition of the retinae proper. It is not clear whether there is any inhibition of the higher stations on the visual pathway during retinal rivalry. For the sake of simplicity, we shall speak of the inhibition between the retinae etc. without always mentioning this latter possibility.

These objects are so simple that in the normal state there is usually no difficulty in deciding which is the dominant one. Fig. 2 is an attempt to illustrate schematically the different degrees of rivalry or the different depths of suppression which are observed. Actual observation of fig. 1 with a stereoscope will, of course, give a much better idea than any illustration or explanation.

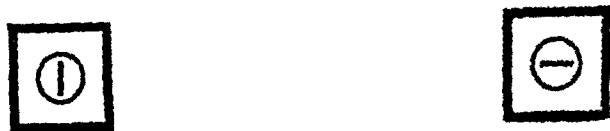


Fig. 1.

At the point of the crossing, the dominant bar is surrounded by a white or gray halo suppressing at least part of the subjugated bar. The depth of suppression can be judged from the amount of the subjugated bar which is invisible. If suppression is weak, there may be only a narrow gray fringe surrounding the dominating bar while in deep suppression the whole subjugated bar may vanish. Some people under normal conditions show frequent total suppressions of the momentarily subjugated bar while in other persons this almost never occurs. Among those who never show complete suppression there are all transitional cases from those who none the less experience a very marked and lively rivalry to those who perceive only very little rivalry, the subject seeing a complete cross without suppression fringes most of the time. In these latter cases, the subject may or may not have a feeling that one of the bars lies behind the other. This seeing one of the bars sometimes in front of the other and sometimes behind, seems to be the first stage of retinal rivalry. When rivalry completely ceases, as sometimes under drugs, a simple cross with both bars in the same plane is seen.

In our experience, most normal subjects show a brisk rivalry with these objects while only a few show the very weak rivalry just described. These latter also have subnormal frequencies of alternation. Only subjects with a well defined rivalry were used in the present experiments.

Most previous observers agree that attention has no influence on retinal rivalry. It is however possible as HELMHOLTZ has pointed out, to read a page of print through a competing picture. It may be that the eye movements necessary for reading are responsible for this result. With our uninteresting and similarly constructed objects we have never been able to influence the rivalry by conscious effort. Blinking and shifting the point of fixation may cause a change in the state of rivalry but this seems to have no influence in the long run. We have made special control experiments with cocainized corneae in order to be sure of avoiding blinking and the results were identical with experiments where blinking was allowed. Our subjects were therefore allowed to blink freely during the observations.

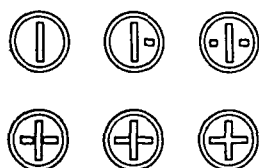


Fig. 2.

Schematic diagram of the different degrees of suppression of the horizontal bar.
 Upper left hand corner: total suppression.
 Lower right hand corner: simultaneous perception.

Rivalry between very simple objects like the two bars used here is characterized by three main variables:

- (1) the average depth of suppression of the subjugated image,
- (2) the relative amount of time during which each of the two eyes is dominant and
- (3) the frequency with which dominance shifts from one eye to the other.

Since our ultimate aim was to find drugs which could be used to reduce the excessive state of retinal inhibition in squint, we were interested in such aspects of rivalry as could serve as measures of the intensity of the state of inhibition. Among these the foremost is, of course, the depth of suppression. Although this can be judged in a subjective manner, it is difficult to express on a really quantitative scale. Perhaps it could be measured by means of an arrangement similar to the one used for studying the light reflex during rivalry (BÁRÁNY and HALLDÉN 1948) but such experiments would certainly be exceedingly laborious. We therefore had to be content with studying the depth of suppression by the subjective method.

In this situation we turned to the other two main aspects of rivalry to see if they could furnish a supplementary measure of the intensity of the state of inhibition between the retinae.

The relative amount of time during which each of the two eyes is dominant was found to serve this purpose.

Assume that the two retinae are presented with two pictures which cannot be fused but possess equal power of suppressing each other. The

two pictures will then dominate about equally often during rivalry and, on an average, for equally long parts of the total time of observation. A drug affecting the amount of reciprocal inhibition could then only affect the other two aspects of the rivalry phenomenon: frequency and depth of suppression. Thus, in a state of perfect balance between the two eyes, changes in the degree of inhibition are not easily measurable.

The matter becomes different if the two retinae are not in balance. Assume for instance that two equal eyes are presented with markedly different pictures, one of them bleak, the other one rich in contour or contrast. There will then be an imbalance in retinal rivalry, the stronger picture tending to dominate most of the time.

The imbalance implies that the inhibition acting on the retina of the "strong" side is relatively weaker than that acting on the retina of the "weak" side. If now a drug reduces the degree of reciprocal inhibition between the two retinae, it will naturally abolish the weaker inhibition before the stronger one. *But this will cause the "stronger" picture to dominate even more than before.* The instants when the "weak" picture is able to suppress the "strong" will be even rarer. Thus, if from the start there is an imbalance between the pictures presented to the two retinae, any change in the level of reciprocal inhibition will be discernible as a change in the degree of imbalance.

The third main aspect of retinal rivalry, the frequency of alternation, did not look promising. However, even at an early stage of the investigation, we observed a marked reduction of the frequency of alternation under the influence of ethyl alcohol. To explain what seemed to be a correlation between depth of suppression and frequency of alternation we went to the literature. We found that LAWAETS in 1939 had discovered a correlation between frequency of alternation and degree of imbalance in retinal rivalry. He achieved a shift in dominance relations by rotating his objects, two crossed bars as in our experiments, so that one of them approached the vertical position, while the other became more horizontal (in this experiment the angle between the bars was 90°). Since the vertical bar tends to dominate, a marked degree of imbalance was obtained. The frequency of alternation fell with increasing degree of imbalance. In another experiment LAWAETS studied the frequency of alternation during the transition from rivalry to fusion. With the angle between the two bars at 15° , fusion was possible only when the bisecting line of the angle was nearly vertical. With the bisecting line horizontal the frequency of alternation was rather high. Now the position of the bisecting line was changed, step by step approaching the vertical. The frequency gradually decreased, as fusion was approximated. There is of course a great difference between fusion and a cessation of rivalry caused by drugs, but one thing they probably have in common, in both conditions the phasic inhibition of one eye by the other must be reduced, as compared with a state of marked rivalry.

In our experiments, we therefore studied the influence of the drugs on the degree of imbalance between two unequal pictures as well as

on the frequency of alternation and on the subjectively perceived depth of suppression.

2. Actual Arrangement of Experiments.

The subjects (laboratory workers, medical students) were seated before a major haploscope (Synoptiscope). The two eyes were presented with the objects illustrated in fig. 1. These were lantern slides reproduced from a large-scale drawing. The circle was seen under a visual angle of 115'. The objects were observed against opal glass screens illuminated from behind by 3 watt flash lamp bulbs run below the rated voltage. The illumination and the position of each object could be individually adjusted. Those subjects who were used to wearing spectacles for correction of their refractive errors were provided with corresponding lenses in the haploscope. For other subjects the images of the objects were placed at infinite distance.

The subject controlled a telegraph key. The duration of the signals and the number of signals were recorded by means of a HRP's chronoscope and a magnetic counter. In some early experiments a tape recorder was used instead.¹

Observations were made in runs of about 70 seconds interspaced with resting periods of about 4 minutes. In alternate runs the subject signalled the dominance of the horizontal and the vertical bar. The key was kept down as long as the bar in question dominated. The subject started each run by observing and signalling a few seconds before the recording equipment was cut into the circuit. The actually recorded period thus embraced the final 60 seconds of a period of about 65—70 secs. The subject had no influence on the exact moment of the start of the recording.

As is well known most people have one dominant eye. Furthermore, it is well known that vertical contours tend to dominate over horizontal contours (PANUM 1858). In the preliminary experiments carried out with each subject, these facts and the possibility of adjusting the illumination of each object separately were used in order to obtain a suitable degree of imbalance between the two pictures. Usually we tried to find a starting level where, under normal conditions, the vertical bar dominated approximately 40 seconds out of every run of 60 recorded seconds.

The haploscope employed allowed of the independent adjustment of the position of the two pictures so that fusion of the circles of the two objects could take place without any effort. During the drug experiments there often was a trend towards esophoria, as previously noted by COLSON (1940) for the case of alcohol and it was frequently necessary to adjust the position of the objects to the changing angle between the visual axes.

¹ With the arrangement used, the number of complete cycles (dominant + suppressed) was counted. The frequency of alternation referred to in the following is the number of such whole cycles per minute.

Most of the normal drug experiments followed the same pattern: During the first hour or so some 10 runs were made in the normal state in order to obtain a control level. Then the drug was administered and after a small pause the experiment was continued. With the exception of the authors (subjects EB and UH) none of the subjects knew the purpose of the experiments or the results to be expected. They were told to make spontaneous comments as soon as something special happened.

The drugs given per os were dissolved or suspended in tap water. The experiments (with some exceptions noted in the text) were made on an empty stomach, about 4 hours after the last meal.

3. Treatment of Experimental Data.

Data obtained from experiments of the kind described are of two kinds. There are the protocol notes covering the experience of the subjects as to depth of suppression and intensity of rivalry. Then there are the numerical data derived from the recorded signals of the subjects.

Condensation of the protocol notes would perhaps have been possible by means of some sort of code. But since the subjects were unaware of the effect sought for, their observations were not phrased on the same pattern and they would need reinterpretation in order to fit into a schematic scale. We have therefore, reluctantly, given the relevant points from the protocols in the form of case histories of all experiments where protocols were kept. Even if the presentation lacks perspicuity the facts are at least undistorted.

The numerical data, on the other hand, have been condensed into graphs. Every complete experiment yielded two series of figures; dominance duration figures for the vertical and the horizontal bars respectively and frequencies of alternation obtained during vertical and horizontal runs respectively. The treatment of these primary figures was based on the following considerations:

We are faced with the problem of combining the data from the two series of runs "vertical" and "horizontal". Obviously, the figures in these series stand in a kind of reciprocal relation to each other; if the vertical bar dominates much, the "vertical run" will give high figures V and the "horizontal run" low figures H. The simplest way of combining the information from the two series is to take the arithmetic means between the V and the "not H".

While the V are simply the recorded durations of vertical dominance for instance in seconds, the "not H" are equal to 60 minus H, since each recorded period embraced exactly 60 seconds.

Now, the runs were made with approximately equal intervals. Each figure belonging to "vertical" is surrounded by two figures derived from "horizontal" runs and vice versa. There is no reason why any one of the two surrounding runs should be preferred in calculating the means. Therefore, the logical thing to do is to calculate the successive means of all experiments. In this way, each figure for "vertical dominant", V, is combined in two means with two different figures for

"horizontal not dominant", 60—H, and vice versa. The procedure obviously is analogous to the smoothing of a distribution by successive means, only in the present case the object of the mathematical manipulation is not the elimination of random variability.

For reasons of symmetry the same procedure was applied to the frequency figures too.

Finally, from the V and H-values the sum $V + H$ was calculated in successive steps. If there were no transitional state, this sum would oscillate around 60 and the amplitude of the oscillations would be a measure of the variability of the recording. Now, there is of course a transitional state between vertical and horizontal dominance and the oscillations usually take place around a value which generally differs from 60. Their amplitude, however, is still a useful measure to watch.

Results.

1. The Normal Course of Retinal Rivalry in Prolonged Experiments.

With rest periods of 3—5 minutes between the 1-minute runs there is no systematic change in either frequency of alternation, depth of suppression or degree of imbalance over periods of several hours. Fig. 3 shows the first training run of the subject P. Z. and a long control run of U. H. as typical examples which give an idea of the degree of precision attainable in a completely untrained and a highly trained subject. If the rest periods are inadequate and fatigue ensues, the inhibition seems to diminish. This matter is more fully discussed in a later part of the paper.

2. Retinal Rivalry under the Influence of Depressant Drugs.

Our first experiments were made with ethyl alcohol because this drug is generally recognized to "remove inhibitions". Alcohol turned out to have a profound influence on retinal rivalry, sometimes completely abolishing it, but the side actions with the necessary doses were so strong as to make many of our recordings worthless. We then tried a number of other depressant drugs and found these to exert the same effect as alcohol on the phenomenon of retinal rivalry, while their general effect on the ability to signal the phases of rivalry was much less disturbing. A general survey of the experiments made is given in table I and on page 10 the curves summarizing the quantitative information derived from the experiments are reproduced.

Amytal. 0.45 g sodium amytal was given to the subject P. Z. 3.5 hours after an ordinary meal. P. Z., who under normal conditions perceives a distinct rivalry with frequent total suppressions of the subjugated bar was completely ignorant of the effect sought for. The experiment was his second drug experiment, in the preceding one he had been given morphia which, in his case, had no definite effect on the depth of suppression. As always, if not specially noted, his observations were offered spontaneously, without suggestive questions from the experimenter.

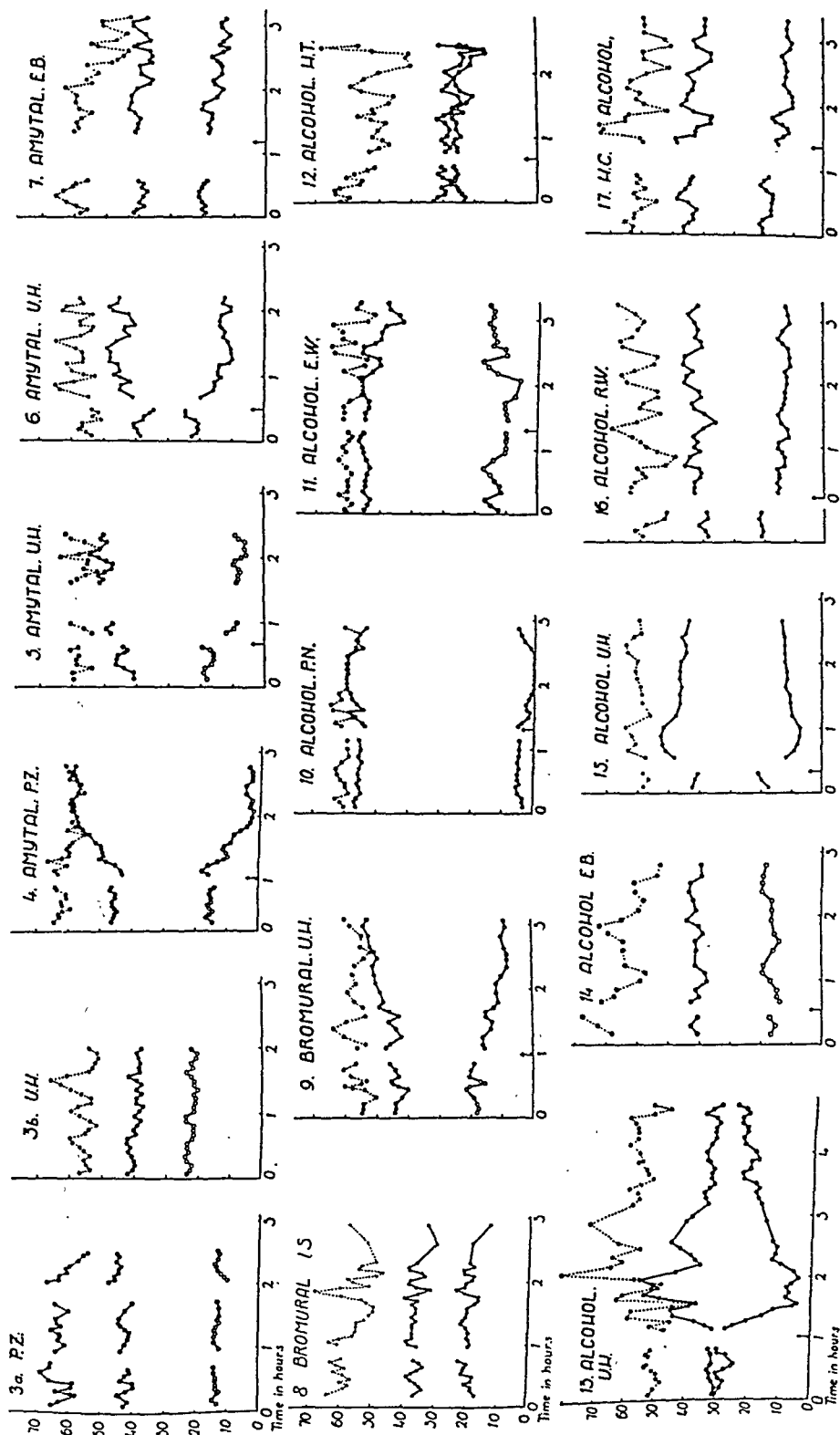
14 minutes after ingestion, he felt slightly intoxicated. At 25 minutes he had slight walking difficulties but still did not say anything about the rivalry experiment. At 37 minutes he said: The two bars now form a complete cross. I signal that bar as dominant which is seen clearer. At 43 minutes his speech was a little indistinct and his gait staggering. 47 minutes: Sleepier and sleepier. Still a complete cross. 74 minutes: Some alternations clearly perceptible but still a complete cross.

Thus, in this subject, amytal almost completely abolished retinal rivalry. Under normal conditions this subject never sees a complete cross but always a brisk rivalry with suppression of smaller or larger parts of one or both bars.

The increase in the degree of imbalance and the reduction in the frequency of alternation caused by the drug are seen in fig. 4.

The same effect was elicited by 0.45 g in the subject U. H. As early as 8 minutes after ingestion there was a diminution in the depth of suppression. 16 minutes: Rivalry gets weaker. There are no longer any total suppressions. Feels slightly drunk. 26 minutes: No rivalry. Cannot decide if the vertical bar dominates the whole time or if there is simultaneous perception. No fluctuations visible. 31 minutes: A little dizzy. No rivalry, as before. 46 minutes: Very little rivalry. The vertical bar, surrounded by a white halo is perceived slightly proximal to the horizontal one. At the boundary between halo and horizontal bar there is a little fluctuation. 56 minutes: Simultaneous perception has ceased, there is rivalry but no total suppressions occur. Two minutes later: The rivalry consists in the two bars exchanging position, the dominating one is seen proximally. At 100 minutes, when the experiment was concluded, there still was only weak rivalry.

Thus, in this case too, a strong dose of amytal caused complete cessation of rivalry and the effect on degree of imbalance and frequency of alternation was unambiguous (fig. 5).



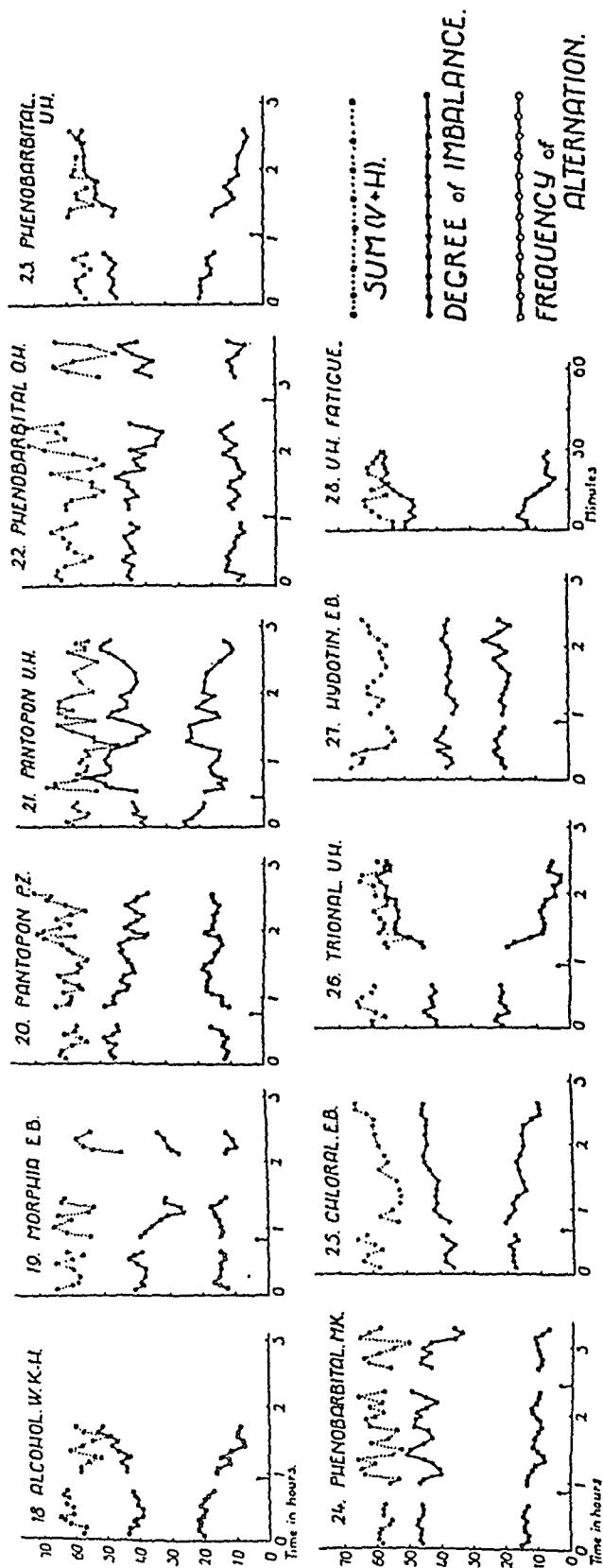


Fig. 3-28.

The meaning of the different curves is indicated on the samples in the lower right hand corner.

With the exception of fig. 28, the abscissae are always hours. The arrows on the abscissae indicate when the drugs were administered.

The "degree of imbalance" is measured by the number of seconds per minute during which the vertical bar is dominant.

In two cases we tried the effect of the normal adult hypnotic dose, 0.3 g. With this dosage, the subjects felt sleepy but not dizzy or intoxicated. The effect on rivalry was less marked. In an experiment on U. H. the alternations became indistinct after 21 minutes but there never was any simultaneous perception. There was a distinct effect on imbalance and frequency (fig. 6).

The same dose in E. B. after 35 minutes gave simultaneous perception for short periods. These periods increased and after 66 minutes there was almost no rivalry during one run. In subsequent runs there was rivalry of slight degree with long transition periods where it was impossible to decide which was the dominating bar. However it never amounted to complete cessation of rivalry. There was, as usual, a moderate reduction in the frequency of alternation, while the imbalance, contrary to expectation, also seemed to be reduced (fig. 7). This is, however, easily explained. The curve of the sums $V + H$ shows a corresponding decrease, indicating an increase in the transitional state between vertical and horizontal dominance.

These experiments are typical examples of the results obtained with depressant drugs.

Bromural. A dose of 1.2 g suspended in water was given to a subject I. S., who had never made any drug experiment before. 40 minutes afterwards, he began to see both bars simulatenously as a complete cross. This went on for the two hours during which the experiment was continued. Some fluctuations persisted however during the whole experiment. He did not explain spontaneously what kind of fluctuations he actually perceived and for fear of suggestion we did not press the point. He did not feel intoxicated, only sedated.

In this experiment there was no certain decrease in frequency of alternation, nor any increase in degree of imbalance (fig. 8). The curve of sums $H + V$ shows that the subject signalled rather irregularly. In this case, as in the case of E. B., fig. 7, there was a marked reduction in the sums $H + V$ under the influence of the drug, indicating an increase in the transitional state between vertical and horizontal dominance. The subject, obviously, was unable to make up his mind how the transitional state was to be interpreted. This explains the irregular curve and the lack of a change in imbalance.

The same dose was taken by U. H. After 20 minutes the sedative effect was noted. Rivalry began to be affected at 33 minutes, when

the alternations became less clearly marked. 42 minutes: Not much rivalry, almost simultaneous perception. 53 minutes: Sometimes simultaneous perception. There is no suppression fringe around the dominant bar, but the dominant bar is perceived as lying proximally and there is some rivalry, the bars exchange position. 96 minutes: Simultaneous perception of both bars but "position rivalry" goes on. 129 minutes: As before. Experiment stopped. Much less intoxicated than after 0.45 g amytal. No motor incoordination.

The effect on degree of imbalance and frequency of alternation was evident (fig. 9).

These two experiments clearly demonstrate that even at a dosage only 20 % above the average hypnotic one, bromural causes weakening and almost cessation of retinal rivalry.

Ethyl alcohol. As already mentioned, this was the first drug tested. Most of our experiments with alcohol had already been made when we discovered that rivalry could cease and simultaneous perception ensue under the influence of the drug. Previously, we had attributed all complaints from the subjects that signalling rivalry was difficult etc. to their reduced power of attention and we did not annotate these complaints regularly. But during the 8:th experiment the following happened.

P. N. had from the beginning a very marked dominance of the vertical bar and a very low frequency of alternation. His weight was 72 kgm and he was given 162 ccm 33% alcohol 9 hours after the last meal. Summarizing his experience of the control runs immediately preceding medication he said: The horizontal bar disappears completely for long periods, while the vertical one is but seldom completely suppressed. As early as 10 minutes after ingestion of the drug he began to see "both bars equally well" while he still did not feel intoxicated. He signalled a few alternations. At 20 minutes, he began to feel intoxicated. At 37 minutes he stopped signalling any alternations. When asked what was the matter he told there were no fluctuations at all, both bars were visible the whole time, the horizontal one a little weaker than the vertical. This condition lasted till 65 minutes. At this time, the horizontal bar began to fluctuate a little but never disappeared completely. At 71 minutes, the horizontal bar for the first time disappeared again completely for a moment and at 95 minutes, when the experiment was finished, the rate of alternations was approximately the same as before the drug was given.

Table I.

In the three "Effect" columns, 0 indicates absence of any effect, — a reduction and + an increase in the variable listed.

Fig.	Subject	Date	Drug	Dosage	Effect on			Notes
					Depth of suppression	Degree of imbalance	Frequency of alternation	
3 a	P. Z.	13/3 47	—	—	0	0	0	
3 b	U. H.	4/4 47	—	—	0	0	0	
4	P. Z.	13/2 47	Amytal	450 mg	—	+	—	
5	U. H.	18/2 47	"	450 mg	—	+	—	
6	U. H.	24/2 47	"	300 mg	—	+	—	
7	E. B.	8/2 47	"	300 mg	—	—	—	
8	I. S.	9/4 47	Bromural	1200 mg	—	0	0	increase of transitional state subject signalled irregularly
9	U. H.	23/2 47	"	1200 mg	—	+	—	
10	P. N.	15/10 46	Alcohol	162 cc	—	+	—	
				33 %				
11	E. W.	17/10 46	"	163 cc	+??	0	0	
				33 %				
12	H. T.	24/10 46	"	203 cc	—	0	—	
				33 %				
13	U. H.	31/10 46	"	225 cc	—	+	—	
				33 %				
14	E. B.	3/10 46	"	225 cc	Not noted	0	0	
				33 %				
15	U. H.	3/10 46	"	225 cc		+	—	
				33 %				
16	R. W.	5/10 46	"	225 cc	"	+	—	
				33 %				
17	H. C.	9/10 46	"	142 cc	"	—	—	
				33 %				
18	W. K.	14/10 46	"	138 cc	"	+	—	
				33 %				
19	E. B.	28/1 47	Morphia	20 mg	—	—	0	
20	P. Z.	18/2 47	Pantopon	20 mg	(—)	—	+	
21	U. H.	1/2 47	"	20 mg	+	complicated curve		
22	O. H.	22/3 47	Pheno-barbital	450 mg	0	0	0	probably delayed re-sorption
23	U. H.	15/3 47	"	300 mg	—	+	—	
24	M. K.	27/3 47	"	450 mg	—	0	—	
25	E. B.	19/4 47	Chloral	2000 mg	—	+	—	
26	U. H.	20/4 47	Trional	1000 mg	—	+	—	
27	E. B.	24/3 47	Hydotin	600 mg	0	0	0	
28	U. H.	28/2 47	Fatigue		?	+	—	

Fig. 10 shows how the degree of imbalance rose and the frequency of alternations fell until rivalry was completely abolished, and how they later returned to nearly normal values. During the whole experiment the degree of intoxication was only slight.

After this experiment we were of course intent on finding the same effect in other subjects and above all with other drugs. From the point of view of therapeutical applications, alcohol has obvious disadvantages and when it was discovered that amytal had the same effect we stopped working with alcohol altogether. Before this was done, however, three more alcohol experiments were made.

In the first of them, on subject E. W., a dose of 163 ccm/72.5 kgm 33 % alcohol 4 hours after a meal elicited no reduction in the intensity of rivalry, as far as the subject told us. At 30 minutes there was a remark to the effect that rivalry was more distinct than before and at 94 minutes a similar remark.

In this experiment, fig. 11, we had chosen an unusually high degree of imbalance as a starting level, hoping to be able to reproduce the successful preceding experiment. As the frequency of alternations was none the less rather high, the inhibition in this case was probably stronger than usual. This might explain the negative result. There was nothing unusual about his intoxication, as far as noticed during the experiment. It was only slight.

Subject H. T. was given 203 ccm/90 kgm 33 % alcohol 5 hours after a meal. He felt intoxicated almost immediately. After 55 minutes he saw both of the bars complete and with equal clarity for part of the time. The same observation was made at 93 minutes.

A starting level with the two retinae in balance had been intended and very nearly obtained in this case. Thus, no effect on the degree of imbalance was expected. Fig. 12 shows that very little, if any, change occurred. The frequency of alternation was reduced, but not greatly. Intoxication was only slight.

In the subject U. H., who in two previous alcohol experiments had had no similar experience, a strong dose of alcohol, 225 ccm/72 kgm 33 % on an empty stomach elicited a very marked effect. Some of the large number of notes made follow:

14 minutes: Begins to feel intoxicated and has some difficulty in signalling correctly. 29 minutes: Rather more intoxicated. 34 minutes: "Crazy experiment. I saw both bars simultaneously and no real alternation. There were no defects in either bar, the cross was complete but there were alternations with regard to position. I signalled the proximal bar as dominant. Signalling was inaccurate and there undoubtedly was simultaneous perception." 44 minutes: "Exceedingly difficult to signal. There is no real difference between the vertical and the horizontal bar, one believes the horizontal

bar is subjugated and discontinuous at the point of crossing but it is difficult to grasp the situation." Feels very intoxicated. 51 minutes: Position rivalry only. Simultaneous perception. 55 minutes: Complete cessation of rivalry. No distance between the objects. 61 minutes: As before. No clue as to dominance. 70 minutes: Begin of rivalry again. — Rivalry was very weak, however, and after half an hour there were a few runs with simultaneous perception and only position rivalry again. Then the drug effect began to wear off and the different stages were passed in inverted order. When the experiment ceased 229 minutes after the ingestion of alcohol, there was still some difficulty in defining the alternations.

In this experiment, too, a starting level with the two retinae in balance was intended but not so nearly attained as in the preceding one. With the strong dosage used, the effect on degree of imbalance and frequency of alternations was distinct (fig. 13).

In the following we will describe the first experiments with alcohol, made before we realized the possibility of having the subjects report the subjective changes in rivalry. The first four experiments will be left out, however, as in two of them the recording of the degree of imbalance was made in a very primitive way and because in none was the frequency of alternations recorded.

Fig. 14 shows an experiment in which the starting level was taken in only four runs. The dosage, 225 ccm 33 % ethyl alcohol on an empty stomach, proved too strong and the subject E. B., 83 kgm, was too intoxicated to be able to signal reliably. This explains why the experiment fails to show significant changes.

Fig. 15 shows the result with the same dosage in another subject, U. H., 72 kgm. A beautifully regular increase in the degree of imbalance and decrease in frequency was followed by a slow return towards the normal values as the intoxication wore off.

Fig. 16 shows the same dosage again in another subject, R. W., 69 kgm. The starting level was taken in only six runs two hours before the experiment. The effect on the degree of imbalance and frequency of alternation was distinct, but considering the high dosage (225 ccm 33 % alcohol) not very great.

Fig. 17 shows an experiment where the result was contrary to the rule. H. C., 63 kgm, was given 142 ccm 33 % alcohol. While the frequency of alternations as usual showed marked reduction, the same happened to the degree of imbalance. We have no adequate explanation for this behaviour.

Fig. 18 shows the results in our only female case, W. K., 65 kgm. She was given 138 ccm 33 % alcohol. There was a typical increase in degree of imbalance and a decrease in frequency of alternation. The experiment had to be discontinued prematurely, however, because the subject vomited.

The experiments described show that ethyl alcohol in sufficient dosage can completely abolish retinal rivalry. In a subject with brisk rivalry, as U. H., the necessary degree of intoxication is rather high, however, and the undesired side effects much more marked than with amytal or bromural.

Morphine. Three experiments were made with this drug.

E. B. was given 2 cg morphine sulphate subcutaneously. 36 minutes afterwards there was a slight diminution of the depth of suppression but no simultaneous perception occurred during the 100 minutes of the experiment. There was a reduction in the degree of imbalance but no influence on the frequency of alternations (fig. 19).

P. Z. received 2 cg of pantopon subcutaneously. When questioned 30 minutes after the injection, he thought rivalry perhaps to be a little weaker than before. This was all, however. Here too, there was some reduction in the degree of imbalance and moreover some rise in the frequency of alternation (fig. 20).

U. H. was also given a subcutaneous injection of 2 cg pantopon. 13 minutes afterwards he felt intoxicated. There was much total suppression of the horizontal bar. (This subject normally sees very little total suppression.) At 16 minutes there was an intensification of rivalry. This was still more marked at 22 minutes, when the horizontal bar was visible only for short moments. At 40 minutes, rivalry began to return towards normal intensity and was clearly perceptible in the rest of the experiment. In some runs, however, the subject complained of difficulties in signalling correctly.

Fig. 21 shows that in this case the changes in imbalance and frequency were complicated. The two variables usually underwent changes in opposite directions, but the intimate course of the curves shows little correspondence with the subjective impressions of rivalry. For the present at least we are unable to explain the observations.

The action of morphine on retinal rivalry obviously differs fundamentally from that of the depressants listed above. From other fields it is well known that morphine causes a mixture of central excitation and depression. This may be the case with rivalry

too. It is interesting to note that GUILLERY (1900) observed an increase in the faculty of fusion under the influence of morphine, while most depressant caused a decrease.

Phenobarbital. This drug was among the last tested. The experiments with alcohol, amytal and bromural, had shown that depressants of three different classes could abolish the reciprocal inhibition of the retinae and we were working with a view to practical applications. A cheap drug with prolonged action would be the best from this point of view and therefore 3 experiments were made with phenobarbital sodium.

In spite of the fact that the drug was given in solution on an empty stomach, one of the subjects O. H. did not feel the slightest effect of 0.45 gm until the next day. In this case no effect on rivalry was noted in the hours following medication, fig. 22. Probably absorption was delayed for unknown reasons. The two other experiments yielded positive results, however.

U. H. received 0.3 gm. After 35 minutes he began to feel a little dazed. At 51 minutes simultaneous perception was first noticed. At 67 minutes rivalry ceased completely for a while. At 80 minutes there were slow shifts in position and sometimes a very narrow suppression fringe around the vertical bar, never around the horizontal one. Fig. 23 shows the decided rise in degree of imbalance and reduction in frequency. — Thus, the effect was the same as with amytal, bromural and alcohol.

M. K. was selected for his very brisk rivalry and clearcut total suppressions of the subjugated bar. He was given 0.3 gm on an empty stomach. After 26 minutes he felt a little drunk. The first run without any total suppression occurred at 30 minutes. In subsequent runs there were a few, short, total suppressions or none at all. But this was the only difference he noticed. At 95 minutes he therefore was given an additional dose of 0.15 gm. 17 minutes after this he complained of difficulties in deciding which one of the bars was dominant. They had a tendency to appear *simultaneously*. 48 minutes after the second dose he said: Rivalry seems to have a tendency to disappear, it is often difficult to decide if either of the bars is dominant. 52 minutes: Slow, almost imperceptible shifts without decided dominance.

At this stage the subject was informed of the aim of the experiment and asked to describe more exactly what he saw, without bothering to signal any shifts. 57 minutes: There are no shifts of position. The vertical bar is more clearly marked than the hori-

zontal one. When the horizontal bar dominates, there is a white fringe across the vertical one. Sometimes it looks as if the subjugated bar were curved around the back of the dominant one. Rivalry is very weak and undecided.

Fig. 24 shows that the reduction in frequency of alternations was distinct. There was, however, no definite increase in degree of imbalance. In the last four runs there was, on the contrary, a marked reduction. We were unable to give any adequate explanation of these aberrant findings.

Summarizing the evidence presented above with regard to depth of suppression we find the following position:

Rivalry was subjectively weakened or abolished in 4 of 4 experiments with amytal, 2 of 2 with bromural, 3 of 4 with ethyl alcohol and 2 of 3 with phenobarbital. In 1 of 3 experiments with morphine there was a marked increase in rivalry and suppression while there was an uncertain decrease in 2. In one alcohol experiment there was an uncertain increase of rivalry. The negative result of the one phenobarbital experiment most probably depended on delayed absorption.

It might be argued that every one of the drugs was tested at least on one of the authors and that we knew what we wanted to happen. Thus, we might have been victims of autosuggestion. Quite apart from the corroborative evidence presented in the curves, we should like to point out that each one of the above drugs was tested on at least one totally unprepared observer too and the complete agreement as to results was obtained in every instance (with the exception of morphine, where the two authors had different experiences). For what it is worth, we should like to point out that we were quite as surprised as the other subjects when we first experienced simultaneous perception.

The data presented do not allow any conclusion as to the relative efficiency of the different drugs for the therapeutical purposes which we have in view. The only conclusion which seems to be possible at the present moment is that depressants of different classes show qualitatively the same effect and that alcohol has more side actions in effective concentration than the other drugs tested. This conclusion is amplified by single experiments which we have made on ourselves with chloral hydrate and trional.

2 gm of chloral hydrate almost completely abolished the frequent total suppressions observed by E. B. and caused short periods of simultaneous perception. The distinct increase in degree

of imbalance and reduction in frequency of alternations is shown in fig. 25. 1 gm of trional led to complete cessation of rivalry for one run and much reduced rivalry in other runs in an experiment on U. H. As shown in fig. 26 there was a decided rise in degree of imbalance and reduction in frequency, as with the other drugs.

It seems, therefore, that the reduction in depth of suppression is a common effect of widely differing central nervous depressants with hypnotic activity. Now there is one interesting group of depressants which has no or only slight hypnotic activity, namely the hydantoin derivatives used as anti-convulsant drugs. The mechanism of the anti-convulsant action of these drugs is not yet understood. Their cortical action seems to be weak, however. (BÁRÁNY and STEIN-JENSEN 1946.) We tested the effect on rivalry of 0.2 and 0.6 gm of 3-methyl 5.5-diphenyl-hydantoin (hydotin) in the subject E. B. There was no influence on rivalry at all. Fig. 27 shows the curves obtained in the 0.6 gm experiment.

Discussion.

We have previously presented evidence for the view that during retinal rivalry the retinae proper suffer phasic inhibitions (BÁRÁNY and HALLDÉN 1948.) One may therefore ask if the influence of the drugs on the rivalry phenomenon is a retinal effect or if the effect is elicited by action on higher centers.

BERNHARD and SKOGLUND (1941) have demonstrated that ethyl alcohol is able to interfere with inhibitory phenomena in the frog's retina. In these experiments, a few drops of a 10 per cent solution of ethyl alcohol were placed in the opened bulb. The concentrations actually effective may therefore have been quite large, probably much larger than those used by us. It seems hardly probable that in our experiments the main point of attack of the drugs was retinal.

The fact that other disturbances of vision were not noted speaks against a peripheral action. In view of the important rôle that inhibitory phenomena in the retina must play for the normal function of the photopic eye it seems improbable that any serious interference with the retinal inhibition mechanisms could have passed unnoticed.

Thus, while we have no definite evidence, we are inclined to the belief that the effect on rivalry is due to an impairment of the central apparatus for single vision. It has been known for many

years that hypnotic drugs and alcohol reduce the fusion faculty (GUILLERY 1900). If one remembers that diplopia is a prominent symptom in poisoning with these drugs, one is led to the conclusion that the central apparatus for single vision must be relatively vulnerable. In the normal state, this apparatus ensures single vision either by fusion or by suppression. Under drugs, the faculty of fusion is reduced, as was previously known, and it was perhaps only to be expected that the same should be true also for the faculty of suppression, as we have shown above.

This view, that the main point of action is the central apparatus for single vision finds some support in the fact that the rivalry phenomenon is subject to fatigue. We have seen that in U. H., with pauses of 3 minutes, between runs of 1 minute, there was no change in rivalry over a period of hours (fig. 3). But when in one experiment the pause between the runs was reduced to 1 minute, fatigue ensued. Fig. 28 shows the result of this experiment. The course of imbalance and frequency are seen to be exactly the same as under the influence of a depressant drug. It seems improbable that the retinal mechanism for inhibition should be so easily fatigued. On the other hand, the central mechanism for single vision must be relatively easily fatigued, as clinical experience with the heterophorias shows.

It may be mentioned that in the subject E. B. we did not succeed in fatiguing the rivalry mechanism.

Finally a remark concerning stereoscopic vision. LIVINGSTONE (1939) has pointed out that retinal rivalry and stereopsis may be correlated in some way. He has found that good stereopsis and brisk rivalry occur together. We therefore have made a few experiments to see if binocular depth perception disappears together with rivalry. This was not the case. We have as yet made too few quantitative experiments to be able to state with certainty if there is any reduction in the ability of depth perception when rivalry is stopped by drugs.

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Summary.

The influence of drugs on retinal rivalry between a horizontal and a vertical bar was studied with the aim of finding pharmaco-

logical agents which might become useful in the treatment of suppression and amblyopia in squint.

Amytal, bromural, chloral, ethyl alcohol, phenobarbital and trional can weaken or abolish retinal rivalry. Under the influence of these drugs, completely simultaneous perception of the two disparate objects may occur. In less pronounced cases only a decrease in depth of suppression of the momentarily subjugated image is observed. Together with the decrease in intensity of rivalry the drugs cause a decrease in the frequency of alternation. If, in the normal state, there is an imbalance between the two competing pictures, one of them being dominant more than half of the time, the degree of imbalance usually increases when rivalry is weakened under the influence of the drugs.

Morphine in one case caused a marked increase, in two other cases uncertain decreases in rivalry. 3-methyl-5.5-diphenyl-hydantoin (one case) had no effect on rivalry.

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Observations on the Hydrogen-Activating Enzymes Present during the Meta- morphosis of Insects.

By

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Metabolism during the metamorphosis of the insects has interested various investigators. A summary of the literature on this subject up to 1929 has been made by NEEDHAM 1929. Concerning the gas metabolism a large number of experiments have shown that the oxygen consumption and the carbon dioxide production give U-shaped curves in relation to the developmental period. During the pupal development the total metabolism seems to pass through a minimum which appears to be determined for individual species belonging to different holometabolic groups of insects. In addition to NEEDHAM's summary (1929) the following works may be mentioned — *Diptera*: FREW 1929, DOBZHANSKY and POULSEN 1935, SQUIRE 1936, HAUB and HITCHCOCK 1941. *Hymenoptera*: BODINE and EVANS 1932, MELAMPY and WILLIS 1939. *Lepidoptera*: BALZAM 1933, SQUIRE 1936, SCHWAN 1940, AGRELL 1947. *Coleoptera*: LUDWIG 1931, SQUIRE 1936. The heat production during the metamorphosis seems to follow the same U-shaped course according to researches by BALZAM 1933 and CRESCITELLI and TAYLOR 1937. In certain cases the variations in the pH as shown by FINK 1925 (Col.), and in the RQ as reported by AGRELL 1947 (Lep.), may give analogous U-curves. There are also several reports on the chemical changes in metabolism during the metamorphosis. The course of the metabolism, as a rule, however, has not been followed during the entire metamorphosis but

comparative determinations of experiments have been made on larvae, pupae, and imagines, respectively. The following more recent reports can also be mentioned in addition to the Needham summary — *Diptera*: FREW 1929, EVANS 1932 (carbohydrate-, fat-, protein-metabolism), HAUB and HITCHOCK 1941 (N-metabolism, carbohydrate synthesis). *Lepidoptera*: RUDOLFS 1926, 1927, 1929 (carbohydrate-, fat-, protein-metabolism), LEIFERT 1935 (excretory metabolism), BABERS 1941 (glycogen metabolism), AGRELL 1947 (fat-, carbohydrate-metabolism). *Coleoptera*: EVANS 1934 (carbohydrate-, fat-, protein-metabolism), BECKER 1934 (fat metabolism). Concerning the researches on the enzyme systems during the metamorphosis the classical work by BATELLI and STERN 1913 should be mentioned and also the following papers: FINK 1930 (catalase activity in *Leptinotarsa*, Col.), WOLSKY 1938 (effect of carbon monoxide on *Drosophila*, Dipt.), BROWN 1938 (enzymes concerning N-metabolism in *Lucilia*, Dipt.), WOLSKY 1941 (dehydrogenase system in *Drosophila*, Dipt.). Concerning the morphological bases of the physiological changes, finally, there are reports on all the above-mentioned groups. PEREZ' detailed work on *Calliphora* 1910, is of special interest in this connection. These earlier investigations will be referred to in the following results.

Material and Methods.

The material used in this investigation was pupae and praepupae of the fly *Calliphora erythrocephala* Meig, which represents an advanced type of holometabolic metamorphosis as a highly specialized dipter. Initial cultures were obtained from the laboratory of Dr. ELLEN THOMSEN, Copenhagen. Imagines were then reared in net cages and fed on water, cane sugar, raw beef, and ordinary household yeast, the latter in order to provide the necessary amount of vitamins. The flies were kept at a room temperature of $+22^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$, and the breeding of larvae and pupae took place at this temperature; the larval stage was about 5 days, the praepupal stage 2 days, and the pupal development 11 days. The transition from praepupa to pupa is not morphologically noticeable. Larvae are considered praepupae when they have stopped eating, have left the meat, and have emptied the intestinal canal. The pupation took place in moist sand to which the larvae made their way of their own accord. Enzyme determinations as well as respiration experiments were usually made every day, starting one day before pupation until the hatching of the pupae. The temperature was in these experiments somewhat higher than the breeding temperature, $+25^{\circ}\text{C}$

$\pm 0.2^{\circ}$ C, because difficulties arose in maintaining the temperature of the water baths at a constant temperature of $+ 22^{\circ}$ C the illumination apparatus causing a gradual heating of the bath above this temperature during the enzyme experiments. The material for the enzyme determinations consisted of approximately 2,500 different tube tests. The following tests were made:

Determination of the gas metabolism.

The determination of the oxygen consumption, the carbon dioxide production and thus also of the respiratory quotient was performed on living pupae with the Thunberg microrespirometer. The method has been described by THUNBERG 1905, JOHANSSON 1920, and AGRELL 1947 among others. In each separate experiment 10 pupae were used with an average weight of about 70 mg. On the identically same pupae enzyme tests were made. The results are given in Fig. 1. Every point on the curve represents the average value of 3 separate determinations.

Determination of the activity of the dehydrogenase systems.

The investigation of the activity of the dehydrogenase system, that is to say the hydrogen-activating capacity, was made with THUNBERG's methylene blue method, according to THUNBERG 1936. In this method methylene blue, called Mb in the following report, is used as the hydrogen acceptor and is decolorized while producing leuco-Mb. The rapidity with which the Mb is decolorized is considered an indication of the rate at which the transport of hydrogen occurs in the dehydrogenase systems. This metabolic rate can thus be measured and so also the activity of the dehydrogenase systems. The rapidity with which the decolorization of Mb occurs is the reciprocal of the time of

the decolorization. In this investigation the value $\frac{1}{\text{time}} \times 100$ has been

used. The capacity of hydrogen transport within the unaffected pupal mass, i. e. the spontaneous activity of the dehydrogenase systems present, is determined in the following manner. Each THUNBERG tube was filled with 1 ml. buffer solution and 0.5 ml. Mb-solution. One pupa was crushed with a glass rod in each tube which was then evacuated for one minute to remove the oxygen and placed in a water bath of $+ 25^{\circ}$ C. The time required for complete decolorization was noted. The Mb-solution used had a concentration of 1:50,000. The buffer was 0.067 M potassium-sodium phosphate of $\text{pH} = 7.5$. The optimum pH for spontaneous decolorization seems to lie around 8. The water bath was of the type described by JOHANSSON 1931, in which a spiral-shaped rotor was used for the stirring. A possible source of error due to the fact that the evacuation time was a varying part of the decolorization time, was excluded by direct correction. The results of these experiments are given in Figs. 1 and 2. Each point of the curves represents the average value of 10 separate tube tests. In the experiments according to Fig. 1, all the determinations of the gas metabolism were performed on the identically same individuals, first the determination of the gas metab-

olism *in vivo* and half an hour to one hour later the determination of the spontaneous activity *in vitro*.

Investigations have been carried out, too, on the effect of various organic substances, which might act as hydrogen donors for the various dehydrogenases, on this normal metabolism. Then 0.5 ml. of the phosphate buffer of the preceding experimental series was substituted by a 0.5 ml. donor solution with a concentration of 0.1 M. The actual donor concentration at the beginning of each experiment was thus at least 0.033 M, the total volume of solution always being 1.5 ml. The substances used will be reported in the part of this investigation dealing with the results. Only potassium salts of the organic acids have been used with the exception of the lactate which has been used as lithium lactate. The substances tested were chemically pure and as freshly made as possible.

The addition of a substance which can act as a hydrogen donor for one of the dehydrogenases active *in vitro* will cause an increase in the Mb-respiration, that is in the hydrogen-activating capacity when compared to unaffected controls, as indicated by a shorter decolorization time, and consequently an increased decolorization rate, provided that the dehydrogenase system in question is not saturated beforehand with this donor in the form of a spontaneous donor. The increase in the metabolic rate after the addition of a certain substance leads us to conclude that the corresponding dehydrogenase enters into the reaction mass of the pupal material examined, and further that the enzyme system in question is not saturated by this spontaneous donor. Such determinations have in this investigation been made for some 30 different substances.

An investigation of the quantitative effect of these substances during the whole metamorphosis is of interest. It is disputable how such a quantitative change in the metabolic rate should be expressed. It might be expressed only as the difference in rate before and after the addition of the donor or in per cent of the unaffected spontaneous activity. If it is assumed that only one of simultaneously active enzyme systems is affected by the donor added, it seems most suitable not to express the difference in per cent of the total rate. Although naturally there always exists the possibility of, for instance, competitive inhibition, or, perhaps, of catalytic activity on the part of the donor added, no pronounced quantitative connection between the various and simultaneous enzyme systems should be presupposed. For the change brought about by an addition of donors the author of this investigation has therefore, in accordance with THUNBERG 1936, used the expression $\left(\frac{1}{A} - \frac{1}{B}\right) \times 100$, where A denotes the decolorization time after the donor addition and B the decolorization time for corresponding controls without the addition of donors. The same expression is used to denote a corresponding inhibition.

The question arises whether this change in the metabolic rate signifies something else besides the presence of an active dehydrogenase system. It cannot be a measure of only the enzyme activity for a constant

enzyme activity with varying concentrations of spontaneous donors gives different values for the change in the metabolic rate upon addition of a donor. On the other hand the change can not merely be a measure of the concentration of spontaneous donors. It should be observed that a constant concentration of spontaneous donors with varying enzyme activity gives different values for the change in activity mentioned. In addition there is the effect of a possibly existing co-enzyme. A change observed in the metabolic rate reflects thus both the effect of the concentration of spontaneous donors and the enzymatic activity, that is to say the activity of the dehydrogenase system. It would perhaps be most correct to call this activity the potential activity as opposed to the actual activity which can be established through the influence of a specific inhibitor. As an illustration a dehydrogenase system may be actually inactive, for example due to the absence of a spontaneous donor. An addition of this donor, however, results in an increased activity; the enzyme system is able to function, *i. e.* it has a potential activity.

The investigation has now been extended to include researches on the quantitative changes in the potential activity of different dehydrogenase systems during the entire metamorphosis. Altogether 16 substances have been investigated in this manner. The results are recorded in Fig. 3 in the form of curves which fluctuate in relation to a zero line denoting no measurable change upon the addition of the substance in question. Each point on the curves represents an average value of 8—10 identical tube tests. The changes observed refer to corresponding curves of the spontaneous activity in Fig. 2, so that hexose diphosphate, lactate, and propionaldehyde refer to curve A in Fig. 2; acetate, propionate, butanol, butanal, butyrate, and aminobutyric acid to curve B; succinate, fumarate, malonate, ethyl alcohol, propanol, and acetaldehyde to curve C; and finally, malate to curve D. The results will be discussed further on in this paper.

The isolated enzymatic activity during the metamorphosis has until now been determined only for the succino dehydrogenase, which does not require the presence of co-dehydrogenase for the reduction of Mb. The following method was used: Two pupae were crushed in a centrifuge tube containing 15 ml. distilled water. The pupal matter was washed by means of a cautious stirring with a glass rod for 15 minutes. The tube with its contents was then centrifuged, 3,600 revolutions per minute, for 10 minutes, after which the washing was repeated. After renewed centrifugation the sediment was transferred to Thunberg-tubes containing 0.5 ml. phosphate buffer, and 0.5 ml. 0.1 M. potassium succinate. Controls in which the succinate was exchanged for the same amount of buffer were prepared in the same way. When calculating approximatively the Mb concentration and the pupal amount previously used, it was observed that the spontaneous activity had now fallen to 2 per cent of the original value. The enzymatic activity is still expressed as the difference between the decolorization rate after addition of a donor and that of the controls. The result appears in Fig. 4. The change in activity expressed in per cent of the control activity gives the identi-

cally same curve. Each point on the curve represents the average of 10 tube tests. An experimental series made at the same time but with somewhat lower dilution in the washings, gave similar result.

The determinations of pH in pupal serum according to Fig. 1, finally, have been carried out colorimetrically by means of a modified Hellige comparator. The separate measurements are therefore fairly uncertain, but the U-shape of the curve might be regarded as definite. The stability of the buffer was also controlled in connection with the determinations of the decolorization time in the Thunberg-tests experiments. An electrometric method was used, which could not be employed in the determination of pH in the pupal serum owing to a lack of micro-electrodes. Tests were carried out at different periods of the pupal development. The pH variation of the buffer was never greater than — 0.2 units and as a rule it was constant or showed only a slight decrease.

Results.

The gas metabolism, oxygen consumption, and carbon dioxide production describes a U-shaped curve during the pupal metamorphosis, Fig. 1. No decrease in the respiratory rate immediately before hatching was observed. The pH-variation also follows a U-shaped course, Fig. 1. The variation in RQ, on the other hand, shows only a tendency to a U-curve, with irregular fluctuations, Fig. 1. The spontaneous activity, *i. e.* the hydrogen-activating capacity of the dehydrogenase system *in vitro* also fluctuates along a clear U-curve, Figs. 1 and 2. A maximum in this activity occurs at pupation. The same maximum in the gas metabolism is probably present but cannot be seen in the results obtained. The measurement of the gas metabolism does not represent the basal metabolism as, contrary to pupae, the praepupae are in motion and consequently the resulting measurements must be considered too high when compared with other values during the pupal period. The variation of the spontaneous activity of the dehydrogenase system is of the same magnitude as that of the gas metabolism. It might therefore be justly presumed that the much disputed U-shaped course of the respiratory curve during the pupal metamorphosis can, at least to a certain extent, be retraced to variations in the activity of the dehydrogenase systems. This is in agreement with similar investigations on *Drosophila* (WOLSKY 1941). Contrary to WOLSKY no absolute accordance was observed here in the courses of the two curves. As appears from Fig. 1 the curves obtained from investigation of identical individuals show different asymmetry: the curve for the gas metabolism is

asymmetric to the left, and the curve for the spontaneous activity is asymmetric to the right. It is possible that the variation in the gas metabolism also depends on the fluctuation of other respiratory enzymes with higher redox potentials. It is also interesting to note

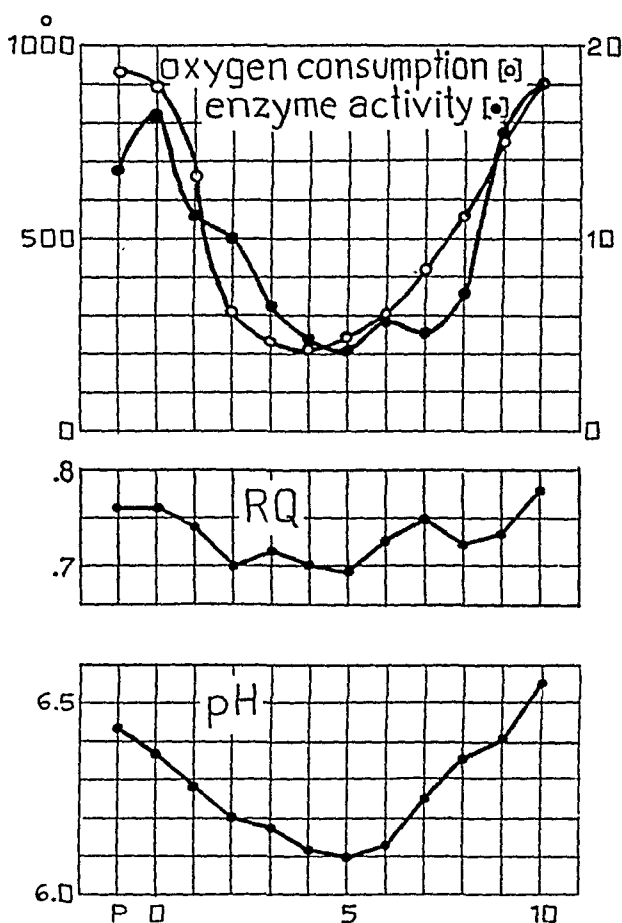


Fig. 1. Oxygen consumption, hydrogen-activating capacity (the spontaneous activity), respiratory quotient together with pH in blood serum during the metamorphosis of *Calliphora*. The ordinate represents ml. O_2 per kg and hour, the magnitude of the Mb-respiration, RQ-units and pH-units, respectively. The abscissa represents the pupal period in days. P denotes praepupa one day before pupation.

the temporary decrease in the spontaneous activity occurring in some experimental series, Figs. 1 and 2, at about 65 % of the pupal time, which does not appear in the curve for gas metabolism.

A number of organic compounds have been observed to stimulate the spontaneous enzymatic metabolism. These observations have only been carried out during the period of minimum metab-

olism, that is to say at about the middle of the pupal time, as preliminary experiments with certain substances had shown an increase in the metabolic rate during this very period. The following substances, enumerated according to their effective power,

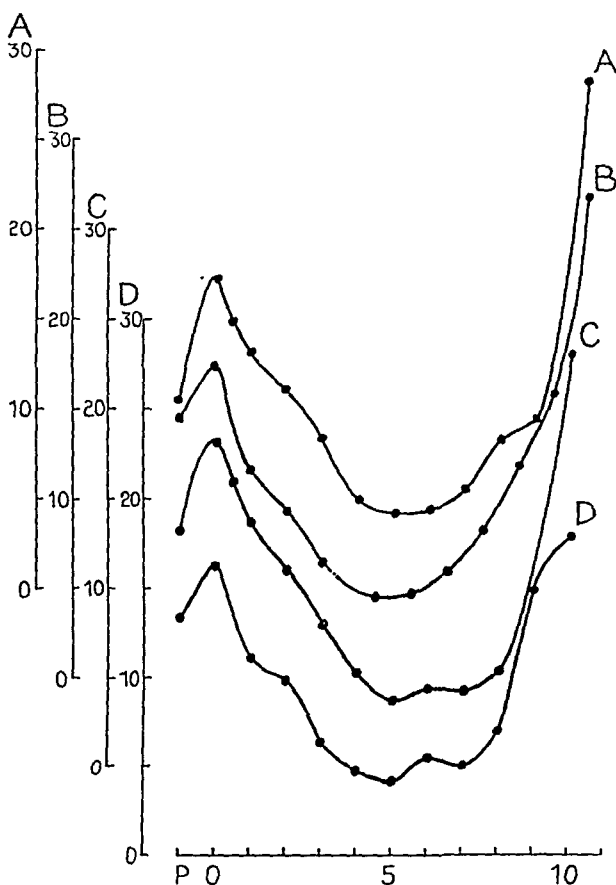


Fig. 2. Hydrogen-activating capacity, *i. e.* the spontaneous activity, during metamorphosis, in four experimental series. The ordinate represents the magnitude of the Mb-respiration. The abscissa represents the pupal period in days as in the preceding fig.

have shown a statistically significant increase in the spontaneous metabolic rate: fumarate, l-malate, glutamate, glycocoll, propanol-1, butanol-1, alanine, formate, valine, succinate, butanal, ethyl alcohol, d-lactate, hexose diphosphate, α -amino-butyric acid, leucine, pyruvate, n-butyrate, β -hydroxybutyrate, propionaldehyde, acetaldehyde, iso-valeral aldehyde, glycollate, acetate, iso-butyrate. There is thus reason to assume that corresponding dehydrogenases are active during the metamorphosis of

Calliphora. Insignificant increase or none whatsoever is observed for the following compounds: xylose saccharose, erythrite, glucose, propionate, capronate, iso-valerinate, caprinate, tartarate. A definite inhibitory effect was produced by the following substances: formaldehyde, malonate, and d-l-asparagine. A possible faint inhibition was caused by methyl alcohol, glycerol, and glyceryl-phosphate. The reason for the inhibitory effect of these substances cannot be established on this preliminary material. The inhibitory effect of malonate, however, confirms the presence of succino dehydrogenase, as malonate is considered a specific inhibitor of this enzyme. When a substance fails to produce an effect, this does indeed not necessarily mean that the substance in question is respiratory inactive. As has been pointed out before, an effect that fails to appear may well be explained by the fact that the dehydrogenase system is already saturated with a spontaneous donor. It will also be demonstrated later on that the potential activity of the enzyme systems fluctuates during the pupal development so that an activating influence during one period may even change into an inhibitory effect during another.

The qualitative determination of active dehydrogenase systems has now been supplemented by investigations on quantitative changes in the potential activity of certain dehydrogenase systems during the entire pupal development. As a measure of these changes the difference in decolorization rate for Mb before and after the addition of enzyme active substance was used, as has been previously mentioned. The results are given in Fig. 3. The fluctuations are obviously systematic. Two different types can be discerned; one displays the same variation as the spontaneous activity. During periods of high spontaneous activity an addition of a donor causes a strong increase in the decolorization rate, but in times of low spontaneous activity only a slight increase. Among the compounds hitherto examined the three alcohols belong to this group. The other substances examined have another rhythmic effect upon the spontaneous activity during the pupal development. The conclusion is that the dehydrogenase systems affected by the substances in question fluctuate periodically in their potential activity during the metamorphosis. Corresponding curves show a succession of maxima and minima, Fig. 3. Three maxima and two minima seem to be the rule. In many cases a minimum means an actual inhibition. The fluctuations seem further to be similarly timed, thus corresponding to generically

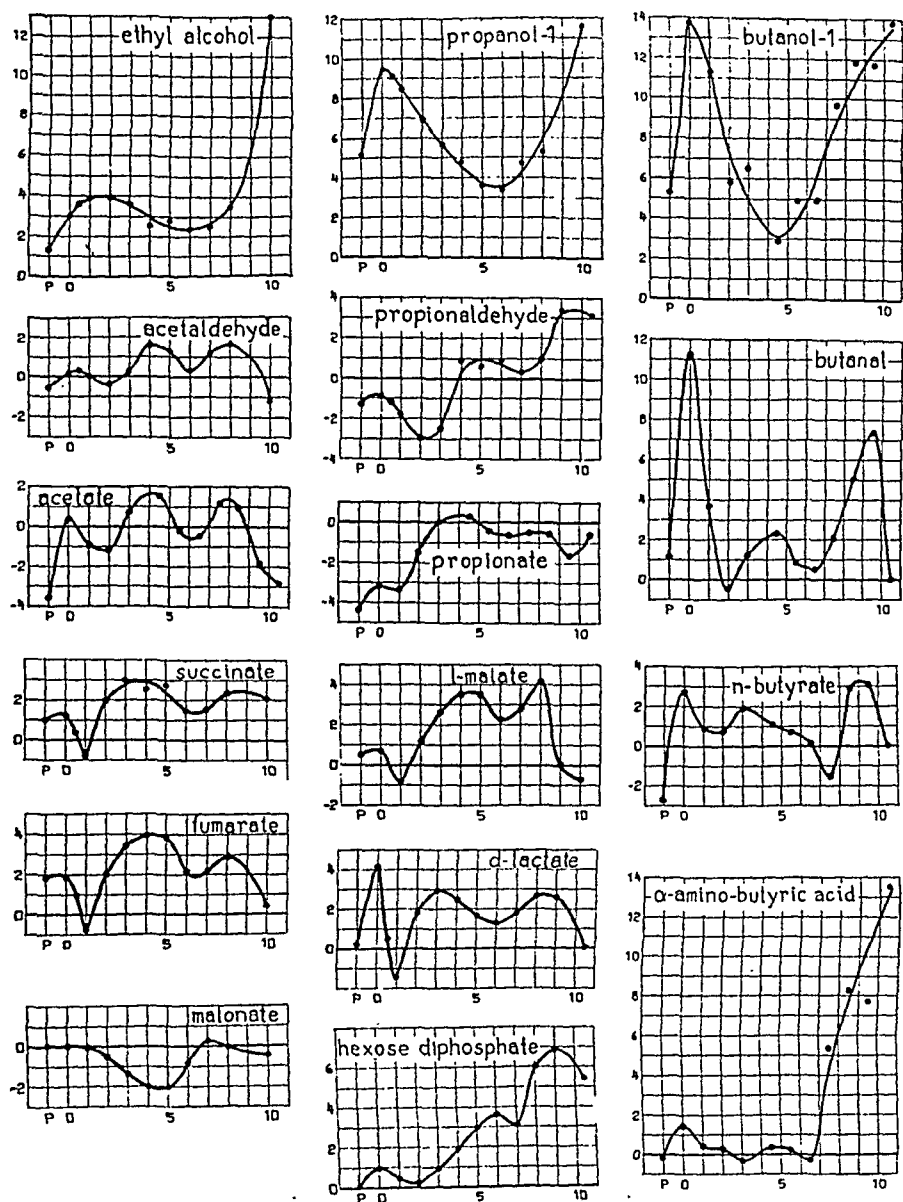


Fig. 3. Changes in the hydrogen-activating capacity, the potential activity of the various dehydrogenase systems, under the influence of various substances during metamorphosis. The ordinate represents changes in the Mb-respiration, the abscissa the pupal period in days as in the preceding figs.

critical periods in the intermediate metabolism during the metamorphosis. Maxima occur immediately after pupation, at about 30 % of the pupal time and at about 70—80 % of the pupal time. Minima occur at 10—20 % and at about 50—60 % of the pupal

time. Among the substances examined only propionate and aminobutyric acid are exceptions: the last maximum does not appear clearly, in the latter case perhaps due to the fact that spontaneous donors, amino acids, diminish rapidly with advancing histogenesis. The inhibitive influence of the malonate will be discussed later.

A determination of the isolated enzymatic activity has, as mentioned before, only been carried out on one enzyme, succino dehydrogenase which does not require the presence of any co-enzyme in order to reduce Mb. The result is represented in Fig. 4.

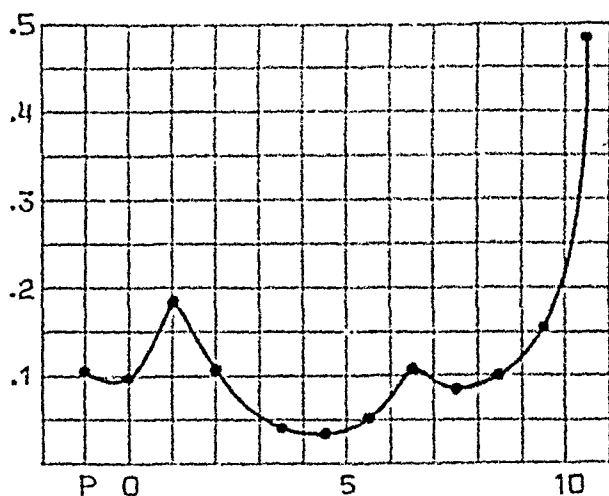


Fig. 4. Changes in the succino-dehydrogenase activity under metamorphosis. The ordinate represents the degree of activity, the abscissa the pupal period in days as in preceding figs.

Evidently succino dehydrogenase undergoes fluctuating changes in its activity during the pupal development. The curve shows alternating minima and maxima, three minima and two maxima. A comparison with the effect of succinate on the spontaneous activity during the metamorphosis, Fig. 3, gives an amazing result. This variation occurs inversely to that of the activity of the succino dehydrogenase; maxima correspond to minima, so that the activity of the succino dehydrogenase is high when the potential activity of the system is low and *vice versa*. After one day's pupal time when the activity of the succino dehydrogenase shows a maximum, an addition of succinate may even prove immediately inhibitive on the spontaneous activity. The fluctuation is not explained by the assumption of corresponding variations in the actual donor concentration, but leads to the assumption of a

formation of some substance with inhibitory effect during the metabolism of succinate, a substance formed only in the unaffected pupal mass and not in the washed substrate. When the succino dehydrogenase activity is high the concentration of inhibitive substance increases but decreases with a lower degree of activity. It follows further that an optimum of the succinate metabolism should exist at a relatively low succino dehydrogenase activity. At the present state of this investigation it is impossible to say which is the inhibitory substance. It is probably one of the oxidation products of succinate, fumarate. KEILIN and HARTREE 1940 have thus demonstrated that oxalo acetate can inhibit the oxidation of succinate.

As has been previously mentioned malonate inhibits the Mb-respiration during a part of the pupal development, viz. during the period of minimal metabolism, Fig. 3. The actual activity of the succino dehydrogenase system is thus reflected by this inhibition, malonate being a specific inhibitor; the system functions therefore only under about 10—65 % of the pupal time. — The fact that the system does not function during the remaining pupal time can be explained by a more or less complete absence of donors. It should be observed that the fluctuation during the latter part of the pupal time is entirely within the limits of error of the method. — The inhibition is percentually high, maximally over 50 %, indicating that more than 50 % of the total Mb-respiration is mediated by the succino dehydrogenase during this period. The great participation of the succino dehydrogenase system in the total respiration suggests the existence of a vast metabolic cycle, in which the succino dehydrogenase system is included, for instance KREBS' citric acid cycle, SZENT-GYÖRGYI's C_4 -cycle, and others. This view is further strengthened by the fact that at 40 % of the pupal time, for instance, an increase in the Mb-respiration on the addition of succinate in the presence of the other spontaneous donors and the co-enzymes, Fig. 3, is about 100 times greater than when these substances have been largely washed away, Fig. 3 and 4. It should be noted that, under the experimental conditions used, washing and centrifuging, a removal of the strongly cell-bound succino dehydrogenase by washing was hardly possible. There is reason to assume, then, that when the respiration during the pupal development approaches a minimum it is supplemented by a previously inactive oxidative metabolic cycle, in which the succino dehydrogenase is an integrating cycle, so to speak, thus

preventing the respiration from reaching too low values. This "succinic cycle" should also affect other dehydrogenases than the succino dehydrogenase and thus other substances than the succinate. In fact, the presence of this assumed cycle can be observed in the change of the potential activity of most of the enzyme systems examined. There exists a maximum occurring more or less at the same time as in the actual function of the succinate system. A peculiarity in the variation of the total unaffected spontaneous activity can now be explained, too, by the assumption of the above-mentioned succinic cycle. After about 55 % of the pupal time there occurs in some experimental series an absolute decrease in the spontaneous activity, Figs. 1 and 2. This temporarily diminished activity, previously mentioned, occurs about the same time as the discontinuance in the activity of the presumed succinic cycle and might perhaps be considered to be caused by the disappearance of this supplementary respiration.

The present investigation deals with the variation in respiratory enzyme systems, in other words, the intermediary oxidative metabolism. It is therefore difficult to compare the results with those obtained by other scientists, which concern the integral metabolism as reflected by the absolute amount of metabolic products. A comparison is made still more difficult by the fact that earlier investigations often do not discuss the successive metabolism during the entire period of metamorphosis but only isolated parts: larva, pupa, imago. A few investigations may be mentioned in this connection, however. Of particular interest are those dealing with the metabolism of diptera, especially of *Calliphora* and related species.

It has been previously mentioned that all diptera hitherto examined show a U-shaped fluctuation of the gas metabolism during the metamorphosis agreeing with that obtained in this investigation. Concerning the biochemical changes during the metamorphosis the following may be mentioned: in a research on *Calliphora* (1905—1909) WEINLAND assumes a fat formation from protein in the larval stage but not during the pupal development. During the former stage a fat combustion takes place as well as an oxybiotic carbohydrate synthesis of protein. Carbohydrate does not seem to be subject to combustion but forms chitin. Anoxybiotically there occurs a gas formation *in vitro* of 2 vol. CO₂ and 1 vol. H₂ from fat. The total amount of N is constant during the pupal time. TANGL's investigations of *Ophyra* (1909)

seem to give fairly similar results. In a report on *Calliphora* (1929) FREW indicates a carbohydrate synthesis from protein during the pupal time especially at pupation. During the latter part of the pupal development he assumes the possibility of a carbohydrate synthesis from fat. The total amount of N is constant. In his investigations of *Lucilia* (1932) EVANS assumes, in agreement with WEINLAND, an eventual fat formation from protein. It is interesting that a transformation of soluble protein-N to insoluble evidently occurs at the assumed beginning of the histogenesis. As the pupal development proceeds excretory-N rises, peptone-N and amino-N fall, and chitin-N remains almost constant. The concentration of the total amount of fatty acids passes a maximum shortly after the beginning of the histogenesis. This maximum seems, however, to affect only the unsaturated fatty acids. In their researches on *Phormia* (1941) HAUB and HITCHOCK seem to have established a carbohydrate synthesis mainly from fat. A maximum of gaseous N-excretion occurs at pupation.

For reasons previously given these partly contradictory results have few points of comparison with the results submitted in this investigation. It is most remarkable that there does not seem to be any clear periodicity in the metabolism of the food substances which, however, is not necessarily evident in the integral metabolism. The following points from the results obtained in this investigation should, however, be stressed: the predominant fat- and protein-metabolism of the pupal development; the biochemically apparently critical period around the pupation; the transformation of soluble protein into insoluble at the beginning of the histogenesis; and the simultaneously occurring maximum in the fat metabolism. These critical periods may concur with periods observed in the fluctuations of the intermediary metabolism. In this connection can be mentioned the observations by AGRELL (1947) on the fixed phases in the gas metabolism of diapausing butterflies, which appear successively during the metamorphosis. These stages are characterized by constant RQ-values, indicating a temporary stabilization of the relation between fat- and carbohydrate-metabolism.

Certain parallels can be drawn, however, to researches on the respiratory-active enzymes. The occurrence of succino dehydrogenase, "succino oxydon" in various insects, also in *Calliphora*, has been established by BATELLI and STERN (1913) by experi-

ments *in vitro*. The oxidation of the succinate, however, is recorded lower for insects than for red musculature, liver etc. of mammals. A parallel is given between the intensity of gas metabolism and succinate oxidation in different insects. Citrate, lactate, acetate, and glucose do not affect the respiration according to these authors. Alcohol oxidase has been noted only in Bombyx-larvae (Lep). These results, which have been obtained by means of respirometric examinations, disagree to a certain extent with the results submitted here. There is, for instance, no parallel between the magnitude of the gas metabolism and the succinate oxidation. Lactate, acetate, alcohols, and citrate, affect the Mb-respiration. A periodically varying effect has been noted for the three first-mentioned substances. FINK (1930) has observed a maximum of catalase activity at the pupation of *Leptinotarsa* (Col.), which stresses the character of the pupation as a biochemically critical period. Of special interest are the researches by WOLSKY (1938 and 1941) on *Drosophila* (Dipt.). The first paper reports a decreased sensibility in the respiration for CO during the period of minimal gas metabolism. The author suggests the reason to be either a destruction-rebuilding of the Warburg-Keilin system or an accumulation-removal of respiratory poisons. His result might possibly be associated with the succinic cycle assumed in this investigation during the period of minimal metabolism. WOLSKY's second publication deals with the quantitative changes in the dehydrogenase systems during the pupal metamorphosis examined by means of the Mb-method. Oxygen consumption and spontaneous activity are said to be directly parallel. An addition of succinate does not alter the results. The author concludes that the dehydrogenase system is saturated with spontaneous donors during the entire pupal development. The results disagree to a certain degree with those submitted in this paper. The parallel between oxygen consumption and spontaneous activity, is, for instance, not complete. The two curves show different asymmetry. Nor is an addition of succinate without effect; a significant and systematic variation in activity is observed.

There are no investigations on quantitative changes in the hormone production during the pupal development. It would be of the utmost interest to establish an eventual correlation between enzyme activity and hormone production. According to histological investigations by VOGT on *Drosophila* (1941) and on *Calliphora* (1943), an increased production of hormone seems to

take place from the ring gland and the corpus allatum respectively, at the beginning of the pupal development.

Finally, concerning the morphological basis of the above-mentioned biochemical changes, several attempts have been made to show that a parallel exists between the U-shape of the respiratory curve and an alternating histolysis and histogenesis. This view is probably now discarded (cf. NEEDHAM 1929 and DOBZHANSKY and POULSEN 1935), one of the reasons being that in carefully examined cases the histolysis is completed at a time when the metabolism is still decreasing. According to PEREZ' thorough researches on the pupal metamorphosis in *Calliphora* (1910), it appears to be difficult to fix any time at which the amount of organized material is larger or smaller than at any other. In other words, histolysis and histogenesis of the various organic systems occur simultaneously. Some results on the dissection of living individuals of the *Calliphora*-pupae examined here may be added to PEREZ' investigation on fixed material.

Shortly after the pupation, at about 8–10 % of the pupal time, the pupa changes its tracheal system. Abdominal tracheae are substituted by thoracal. During this time neither of the tracheal systems seem to function effectively as they are hardly air-filled. At about 60 % of the pupal time the heart starts working and at about 70 % of the pupal time the granulocytes disappear entirely from the blood serum. These data coincide with critical periods observed in the activity of the dehydrogenase systems. It is thus quite possible that the functional capacity of the various organic systems is gradually accumulated to form the total respiration observed in the U-shaped respiratory curve. The immediate cause is, however, to be found in a parallel variation in the activity of the respiratory enzyme systems.

The technical work in this investigation has been carried out by Miss ELSA ROSENGREN. The investigation has been facilitated by grants from the Scandinavian Insulin Foundation and from the Swedish Government Research Board for Natural Science.

Summary.

1) The aim of this investigation has been to study the variation of the intermediate oxidative metabolism during insect metabolism as reflected in the activity of the dehydrogenase systems.

Pupae and praepupae of the fly *Calliphora erythrocephala* Meig. were used and proved to be excellent experimental material for this purpose.

2) The gas metabolism, oxygen consumption, and carbon dioxide production, determined by means of the Thunberg-microspirometer, described a U-shaped curve during metamorphosis. It was shown by the application of Thunbergs' methylene blue method that the experimental animals were carriers of hydrogen-activating enzyme systems. Variations in the hydrogen-activating capacity of the dehydrogenase systems or their spontaneous activity are likewise U-shaped and of the same order of magnitude as that of the gas metabolism. For this reason it is assumed that the U-shaped course of the respiration is, to a certain degree, connected with variations in the activity of the dehydrogenase systems. There is, however, no absolute agreement between the variations in the gas metabolism and the dehydrogenase activity. Variations in pH in the serum also give a U-shaped curve.

3) About 30 organic compounds have been found capable of increasing the hydrogen-activating capacity of the experimental animals *in vitro*. There is reason to assume that corresponding dehydrogenases are active during metamorphosis. Also inhibiting substances are observed.

4) Changes in the hydrogen-activating capacity due to the effect of 16 different substances have been followed during the entire metamorphosis. The results obtained show systematic, periodically occurring variations in activity, which are considered an indication of the change in the potential activity of the respective dehydrogenase systems.

5) The fluctuations in the potential activity seem to occur at definite time intervals and for this reason it is thought that they represent generally critical periods in the intermediary oxidative metabolism during metamorphosis.

6) The changes in the activity of the succino dehydrogenase has been studied during the entire metamorphosis, as well as the inhibitory effect of malonate and the influence of succinate on the spontaneous activity during the same period. The results obtained give reason to assume the existence of a "succinic cycle" during the period of minimal metabolism during the metamorphosis.

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From the Biochemical Institute of the University, Copenhagen.

On the Effect of Copper on Cytochrome Oxidase.

By

GUNNAR STEENSHOLT.

Received 3 September 1947.

It has been known for a long time that copper is one of those metals which, though present only in minute amounts, are of great importance for various chemical reactions in the animal body. Its absence from the diet causes symptoms of disease. It is, however, only in more recent years that copper has been associated with more specific cellular components and with definite chemical processes. In the present note we shall first draw attention to the work of COHEN and ELVEHJEM (1934), who reported that in nutritional anemia of rats the cytochrome content of heart, liver and brain was markedly decreased. The addition of small amounts of copper to the diet of anemic rats restored the normal cytochrome spectrum, while iron did not have any effect. When indophenol oxidase activity of the liver was measured by means of the nadi reagent an analogous result was obtained.

It is well known that more recent work has established the identity of indophenol oxidase and cytochrome oxidase. Moreover, better methods for the determination of cytochrome oxidase activity have been worked out (STOTZ, SIDWELL and HOGNESS 1938). These developments made it possible for SCHULTZE (1939) to repeat and extend the work of COHEN and ELVEHJEM. In the experiments of SCHULTZE rats were made anemic by the method of ELVEHJEM and KEMMERER (1931). For determination of cytochrome oxidase activity the animals were decapitated, and the required organs removed and homogenized. Their cytochrome oxidase activity was then measured by the method of Stotz

et. al. just referred to. In this way liver and heart tissue of copper deficient and of iron deficient rats was studied. It was found that in copper deficiency (when iron was fed) the cytochrome oxidase activity of liver and heart tissue, expressed per unit weight, was greatly reduced. In iron deficiency (when copper was fed) the cytochrome oxidase activity was not reduced, and in severe deficiency it was even increased. It was concluded that copper is essential for the formation and maintenance of cytochrome oxidase activity of liver and heart tissue in rats.

In the work of SCHULTZE copper analyses could unfortunately not be carried out on the tissues under investigation. She points out, however, that it is known from other work that copper deficiency leads to depletion of copper from the organs, particularly from the liver of the rat, while on the other hand feeding of copper can lead to accumulation of the metal in the liver. The problem therefore naturally presents itself whether the cytochrome oxidase activity of tissues is directly due to a copper compound, as was actually suggested by KEILIN and HARTREE (1939). The experiments of SCHULTZE might seem to support this view. However, she rightly points out the necessity of remembering that even severely anemic rats still contain measurable amounts of both copper and iron, particularly the latter. If cytochrome oxidase activity is due to iron catalysis one might interpret the results to mean that copper is necessary, by some mechanism yet unknown, for the formation of the catalyst (analogous to the formation of hemoglobin). Finally one should not forget, as is also pointed out by Miss SCHULTZE, that cytochrome oxidase activity may well be a composite effect of more than one catalytically active substance.

It may be very hard to decide these difficult questions until ways and means of carrying out appropriate isolation studies on cytochrome oxidase have been found. Pending the development of such methods we must look for other procedures that might conceivably be capable of throwing some light on the question. In this connexion attention may be directed to remark by FRANKE (1939—40), who has pointed out that inhibition experiments with substances such as sodium diethyl-dithiocarbamate, which are capable of forming complexes with copper, may well give some information of interest for the elucidation of the rôle of copper in certain oxidation systems. Work of this type does not seem to have been recorded in the literature, at any rate

not as far as cytochrome oxidase activity is concerned. It is the purpose of the present note to report on some experiments in this direction.

Experimental Part.

Our biological material was liver tissue from rats, which had been kept for some time in the laboratory on a diet believed to be sufficient in all respects.

In order to determine cytochrome oxidase activity by the method applied in the present piece of investigation, cytochrome c is required. A sample was prepared in the following way according to KEILIN and HARTREE (1937). A heart was freed from fat and ligaments and cut very finely. The blood was pressed out as well as could be done in a hand press and 1,100 g of the pulp were mixed with 1,100 ml 0.15 N trichloroacetic acid. The mixture was allowed to stand for two hours at room temperature under occasional stirring. The liquid was then pressed out and caustic soda added until the hydrogen ion concentration was brought to about pH 7.0. The mixture was centrifuged for 10 minutes, and 1,250 ml of the supernatant liquid treated with 625 g ammonium sulphate. By this treatment hemoglobin is removed. The precipitate was filtered off, and the filtrate was again treated with ammonium sulphate (5 g of the salt per 100 ml liquid). The mixture was now left overnight in the refrigerator. The next day it was filtered again, and while still cold treated with 1/40 of its volume of 20 per cent trichloroacetic acid. The pH was thereby (as measured by a glass electrode) brought to 3.75. After a while the precipitate was spun down in a centrifuge, a bright red deposit being obtained. It was shaken with 500 ml saturated ammonium sulphate solution and centrifuged again. The red precipitate was suspended in 20 ml distilled water and transferred to a cellophane bag, in which it was dialyzed for two days at 4° C against a 1 per cent sodium chloride solution. After removal from the bag the suspension was shaken with 3 drops of chloroform and filtered. The final result was 29 ml of a clear dark red solution. It was analyzed for iron by a method of LINTZEL (1933). According to KEILIN, cytochrome c contains 0.34 per cent iron, and, accepting this figure, our solution was found to contain 0.155 g pure cytochrome. The whole procedure worked smoothly. Another sample of cytochrome c was obtained from horse heart, which was also worked up by the method described above. Both samples behaved identically in the experiments reported below.

For the determination of cytochrome oxidase activity the method of STORZ, SIDWELL and HOGNESS (loc. cit.) was applied. This is a manometric method, and the ordinary Warburg apparatus was used. The vessels contained an excess of cytochrome c (0.15 ml containing 5.6 micrograms Fe); 0.4 ml semicarbazide hydrochloride solution (neutralized to pH 7.2; final concentration 0.02 M); a suitable amount of homogenized liver tissue (as a rule 0.25 or 0.30 ml); and M/15 phosphate buffer of pH 7.2. Hydroquinone (5×10^{-5} M) in 0.25 ml water was

added from the side chamber after equilibration in the water bath. The final volume was 2.8 ml after addition of the hydroquinone solution in the side chamber. The central chamber contained 0.2 ml of a 20 per cent solution of potassium hydroxide for the absorption of carbon dioxide. A manometer without cytochrome was used as control. The oxygen uptake measured in this manometer is of course the uptake due to tissue respiration, autoxidation of the hydroquinone and oxidation of the hydroquinone by the tissue. Duplicate experiments were always carried out. The manometers were read at 5 minute intervals during half an hour. The tissue suspension contained an amount of tissue of the order of 1 g tissue per 15 to 20 ml suspension. The oxygen uptake then remained within limits convenient for measurement in the Warburg apparatus.

Our main purpose was to investigate the effect of sodium diethyl-dithio carbamate on cytochrome oxidase activity. This was done by adding varying amounts of this compound to the reaction mixtures and comparing the oxygen uptake to that of mixtures to which the substance had not been added. For reasons of economy of space we can quote only a few typical experiments.

In one experiment a Warburg vessel was prepared as described above. Another vessel was prepared in identically the same way, but 0.01 mg sodium diethyl-dithio carbamate were added together with the tissue suspension. At the end of a 30 minutes period the oxygen uptake in two vessels of this type was 140 and 137 mm. Two other vessels without sodium diethyl-dithio carbamate gave the following values for the oxygen uptake: 141 and 137 mm. Repeating the experiment with 0.04 mg of the complex forming compound we found the following oxygen uptakes: 139, 141, 137 and 142 mm. With 0.08 mg sodium diethyl-dithio carbamate we found: 138, 139, 137 and 142 mm.

From the estimates of the copper content of liver (and other organs) given in the literature it can be seen that the complex forming substance was added in quantities of at least the same order of magnitude as those in which copper is present.

The experiments above were repeated with several other rat liver homogenates, with substantially the same results. It was concluded that if sodium diethyl-dithio carbamate has any effect at all, it must certainly be very small and below the limit of error in our experiments.

We also carried out complete determinations of cytochrome oxidase activity in our rat liver suspensions, but the results obtained are of no immediate interest for the problem in hand, and the numerical details are therefore omitted.

It is clear that the results reported above tend to support the conclusion that the presence of copper is not essential for the action of cytochrome oxidase. Hence they contradict to a certain degree the suggestion of KEILIN and HARTREE referred to above. According to our experiments an interpretation of the work of ELVEHJEM and his associates, and of SCHULTZE, may more likely be found along other lines, for instance those indicated in the introduction.

The writer is glad to express his best thanks to Professor R. EGE for generous hospitality and support.

Summary.

It is found that the copper complex forming compound sodium diethyl-dithio carbamate has no, or only an extremely small effect on cytochrome oxidase activity. The interpretation of this result and its bearing on previous work are briefly discussed.

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On the Methylation of Ethanol Amine, Dimethyl Ethanol Amine, Guanidine Acetic Acid and Homocysteine.

By

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The problem of the biosynthesis of choline and creatine has in recent years attracted the attention of a considerable number of biochemists, and a rather extensive literature has grown up subsequent upon the pioneer work of DU VIGNEAUD and his associates. The present writer also made some contributions to these studies; in particular he occupied himself with the *in vitro* synthesis of choline from ethanol amine, and creatine from guanidine acetic acid, with methionine as a methyl donator in a suspension of tissue pulp (STEENSHOLT, 1945, 1946). In this work the increase in the amount of choline and creatine in the reaction mixtures was determined and used as a measure of the rate of reaction of the methylation process. However, in the case of choline, this method suffers from the defect that ethanol amine interferes to some extent with the determination of choline. For dimethyl ethanol amine this interference effect is even more pronounced, as could be expected on account of the similarities of the molecular structures. Moreover, the action of the enzyme choline oxidase in many cases tends to decrease the amount of choline synthesised during the experiment. It appears desirable, therefore, to corroborate the findings described in the earlier notes, by applying methods in which the difficulties just mentioned are, as far as possible, surmounted or evaded. An obvious possibility is to attempt to determine the decrease in the amount of methionine in the reaction

mixtures during the experiment. It is the purpose of the present note to report on some results in this direction.

The same method will also be applied to the methylation of guanidine acetic acid to creatine, and to a verification of some previous results on the methylating properties of etiolated wheat germs.

When methionine acts as methyl donator it is demethylated to homocysteine. There is an experimental basis (feeding experiments on rats) for the assumption that the reverse reaction, *i. e.* methylation of homocysteine to methionine, also takes place in the animal organism. It would therefore seem to be of some interest to study this reaction by *in vitro* experiments, and some results pertaining to this problem will also be reported in the present paper.

Experimental Results and their Discussion.

Biological material. In the experiments on mammalian tissue rat liver was used throughout. As usual, the animals, which had been kept on a diet believed to be sufficient in all respects, were killed by decapitation, and the liver removed immediately afterwards. The organ was then placed on a watch glass and finely divided by means of a pair of bent scissors. In this state the tissue could be conveniently handled and weighed.

In the course of the present work on methylation processes, it was, as already mentioned, considered of some interest to reexamine our previous work on methylation processes in etiolated wheat germs. The wheat used was as before of three different kinds:

- 1) Jubilee wheat;
- 2) Svalöv Skandia wheat;
- 3) Record wheat.

The etiolation was carried out in the ordinary way by placing the germs on wet filter paper in the dark. After 6 to 7 days the etiolated germs were cut into small pieces and carefully ground in a mortar. The resulting mass was used in the subsequent experiments.

Substrates and reagents. The ethanol amine was synthesised by the method of KNORR (1897). This procedure consists in blowing a stream of ethylene oxide through a concentrated aqueous ammonia solution and subsequently fractionating the reaction mixture. Dimethyl ethanol amine was obtained by essentially the same method with ammonia replaced by dimethyl amine. Both procedures were found to go smoothly and to yield products believed to be of a high degree of purity. Commercial samples of both compounds were also available. They were carefully purified by repeated distillations, and were found to behave in exactly the same way as those synthesised by the present writer.

Guanidine acetic acid was synthesised by the method of NENCKI and SIEBERT (1878). The methionine was a Hoffmann-la Roche product, and the homocysteine was obtained from Professor DU VIGNEAUD, of Cornell University Medical School, to whom the writer is greatly indebted for his generosity.

For deproteination a solution of trichloroacetic acid was used. In several cases parallel experiments with a 10 and a 20 per cent solution were carried out with identical results in all cases.

Determination of methionine. For this purpose we have applied the colorimetric method developed by MCCARTHY and SULLIVAN (1941). This method was ordinarily applied in the following form: 5 ml of the solution to be analysed were introduced into an ordinary test tube, and 1 ml of a 14.3 N sodium hydroxide solution was added, followed by 1 ml of a 1 per cent aqueous solution of glycine, and 0.6 ml of a 10 per cent aqueous solution of sodium nitroprusside, with good mixing after each addition. The tube was then placed in a water bath at a temperature of 35–40° C. for 10 minutes. It was then cooled in ice water for 2 minutes, and 5 ml of a mixture of 9 volumes of concentrated hydrochloric acid and 1 volume of 85 per cent phosphoric acid were added, with thorough shaking and mixing during the addition. Shaking was continued for 1 minute after the addition of the acid was completed, and the tube was then cooled in water at room temperature for 10 minutes. Finally the colorimetric determinations were carried out in a Pulfrich photometer in the ordinary way, using a blank obtained by treating 5 ml of water in the way just described.

All our experiments were carried out using McIlvaine's phosphate-citrate buffer.

A typical experiment with ethanol amine as methyl acceptor was carried out as follows. Two flasks, A and B, were prepared with the following reaction mixtures. Flask A contained:

- 0.3 g rat liver pulp
- 0.05 ml ethanol amine
- 56 mg methionine
- 3 ml McIlvaine's phosphate-citrate buffer (pH 7.2).

Flask B contained:

- 0.3 g rat liver pulp
- 56 mg methionine
- 3 ml McIlvaine's phosphate citrate buffer (pH 7.2).

The contents were carefully mixed, and both flasks were then incubated at 37° C in a water bath for 10 hours. 2 ml of a 10 per cent trichloroacetic acid solution were then added, and after stirring the mixture was left standing for 10 minutes. The precipitate was removed by centrifugation, and 2 ml of the clear supernatant liquid were diluted with 20 ml of water. Of this mix-

ture 5 ml were removed for determination of methionine according to the method of MCCARTHY and SULLIVAN. The result was a 15.5 per cent decrease in the content of methionine in flask A compared to flask B.

The above example was chosen at random from the laboratory journal. The experiment was varied by using different relative amounts of methionine and ethanol amine, and by varying the time of incubation from 5 hours up to 16 hours. The results were qualitatively the same in all cases. As an illustration of this we shall quote only one further experiment, in which flask A contained

0.3 g rat liver pulp
0.05 ml ethanol amine
70 mg methionine
4 ml McIlvaine's buffer (pH 7.3).

The content of flask B was exactly the same except that ethanol amine was omitted. The flasks were incubated at 37° C for 5 hours, and their contents worked up as described above. In this case we found a 10.2 per cent decrease in the amount of methionine in flask A compared to that in flask B.

Similar results were obtained when the ethanol amine was replaced by dimethyl ethanol amine. We shall quote only one experiment, since it is sufficiently typical for this part of our work.

The six flasks A, B, C, D, E, F contained 0.3 g rat liver pulp and 4 ml McIlvaine's phosphate-citrate buffer (pH 7.4). In addition flasks A, B and C contained 0.05 ml dimethyl ethanol amine. Flasks A and D contained 25 mg, B and E 50 mg and flasks C and F 75 mg methionine. All six flasks were incubated for 12 hours at 37° C, and after removal from the water bath their contents were worked up and analyzed as described above. On comparing the content of methionine in A and D, B and E, and C and F, we found reductions amounting to 19 per cent, 9 per cent, and 9.5 per cent respectively.

By carrying out similar experiments at different hydrogen ion concentrations, we found, both for ethanol amine and dimethyl ethanol amine, a pH optimum slightly above the neutral point. This agrees with results previously reported for ethanol amine (STEENSHOLT loc. cit.).

The writer was unable to get any reliable information about the relative velocities of methylation of ethanol amine and dimethyl ethanol amine. However, it must be borne in mind that the process

of methylating ethanol amine to choline probably proceeds step-wise, and the disentanglement of the intermediary reactions is therefore a problem the solution of which requires more accurate methods than those available at present. For the time being the question of the details in the reaction kinetics must be left aside, pending the development of refined analytical methods and perhaps also the purification of the enzymes that catalyze the processes in question.

The experiments on etiolated wheat germs were carried out as previously described (STEENSHOLT, loc. cit.), except that the reaction mixtures were analyzed for methionine by the method of MCCARTHY and SULLIVAN. No trace of a methylation process could be detected, and, in corroboration of previous results we may therefore conclude that etiolated wheat germs do not contain enzyme systems capable of catalyzing the methylation of ethanol amine or dimethyl ethanol amine to choline. Our previous work was carried out only with ethanol amine.

We shall finally mention that in separate experiments we investigated the effect of the presence of ethanol amine and dimethyl ethanol amine on the determination of methionine by the method of MCCARTHY and SULLIVAN. With the relative concentrations employed in our work the effect was found to be inappreciable. We found that the presence of the compounds in question tends to increase slightly the amount of methionine found by the colorimetric method, which is an additional argument in favour of the soundness of the conclusions drawn from our experiments.

The same method was then applied to an investigation of the methylation of glycocyamine to creatine. In a typical experiment flask A contained:

0.3 g rat liver pulp
8 mg glycocyamine
50 mg methionine
4 ml McIlvaine's phosphate-citrate buffer (pH 7.4).

The content of flask B was exactly the same, except that it contained no glycocyamine. The flasks were incubated for 8 hours, and the reaction mixtures were afterwards worked up as described above. Analysis by the method of MCCARTHY and SULLIVAN showed a 10.9 per cent decrease in the amount of methionine in flask A compared to flask B.

As regards the effect of the presence of glycocyamine on the

determination of methionine by our colorimetric method, we found, for the relative concentrations employed in our experiments, results quite similar to those just described for ethanol amine and dimethyl ethanol amine.

In further experiments we determined the pH optimum for the methylation process in question. In agreement with previous work (loc. cit.) the optimum was found to lie slightly above the neutrality point.

In studying the methylation of glycocyamine to creatine a rather obvious possibility is to follow the reaction by measurements of the content of glycocyamine in the reaction mixtures during the experiment. On the basis of work by several previous authors DUBNOFF and BORSOOK (1941) developed a micromethod for the determination of glycocyamine in biological mixtures. This procedure is based on a judicious application of the SAKAGUCHI reaction. The writer made several attempts to apply this method to the present problem. However, the method was found to give erratic and often completely erroneous results, and the work therefore had to be discontinued. The cause of the trouble was considered to be the available types of permutite which were found to be unsuitable for the purpose in hand. It was later found that other workers have reported similar findings. In future applications of the method of DUBNOFF and BORSOOK some such modification of the procedure as the one developed by SIMS (1944) will probably have to be applied. This could not be done here since the adsorbent described by SIMS was unavailable.

A further point may be briefly mentioned at this place. ALBANESE, FRANKSTON and IRBY have recently developed a method of estimating methionine in protein hydrolysates and urine (ALBANESE *c. s.*, 1944). This procedure depends on an oxidation of methionine with a mixture of perchloric acid and hydrogen peroxide. The present writer has applied the method to the problems discussed in this paper and obtained results in qualitative and rough quantitative agreement with those found by the method of MCCARTHY and SULLIVAN. However, the method of ALBANESE, FRANKSTON and IRBY was, at any rate in its application to our problems, found to require extreme care in its execution in order to yield fairly reproducible results. The present writer is therefore not inclined to put too much confidence in the quantitative results obtained by this procedure. The numerical details are therefore omitted.

Finally, as was already mentioned by way of introduction, we have made an attempt to investigate by *in vitro* studies the methylation of homocysteine to methionine. Choline and methionine were used as methyl donators, and the experiments were carried out as previously described. It will suffice to report the details of only one typical experiment, in which flask A contained

0.3 g rat liver pulp
25 mg homocysteine
37 mg choline
4 ml McIlvaine's buffer (pH 7.2).

The content in flask B was exactly the same except that choline was omitted. The flasks were incubated for 5 hours, and the mixtures then worked up and analyzed as above. No trace of a formation of methionine could be detected. When in this experiment choline was replaced by 40 mg betaine the same result was obtained. Experiments were also carried out with the double and fourfold amount of methyl donator, and at pH 5, 6 and 8, and finally the series of experiments was worked through with 50 mg homocysteine instead of 25 mg as in the example just quoted. A formation of methionine could not be found in any of the cases.

By measuring, in the above experiments, the increase in choline and creatine simultaneously with the decrease in methionine in our reaction mixtures, it might seem possible to demonstrate quantitatively the relationships governing the transfer of methyl groups from donator to acceptor. Several attempts were made along this line, but sufficiently precise data could not be obtained, and the numerical details will therefore not be further discussed in this paper. It seems likely that further progress along this line must await the purification of the enzyme systems which catalyze the reactions in question. Rat liver pulp is a much too crude mixture to avoid all the side reactions that tend to complicate the situation.

The author is glad to express his best thanks to Professor R. EGE for generous hospitality and support.

Summary.

By means of the colorimetric method of MCCARTHY and SULLIVAN for the determination of methionine in biological mixtures,

an investigation is made of the methylation of ethanol amine, dimethyl ethanol amine, glycocyamine and homocysteine. The results extend and corroborate those of previous investigations.

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From the Biochemical Institute of the University, Copenhagen.

On the Rôle of Glycollic Acid in Biological Methylation Processes.

By

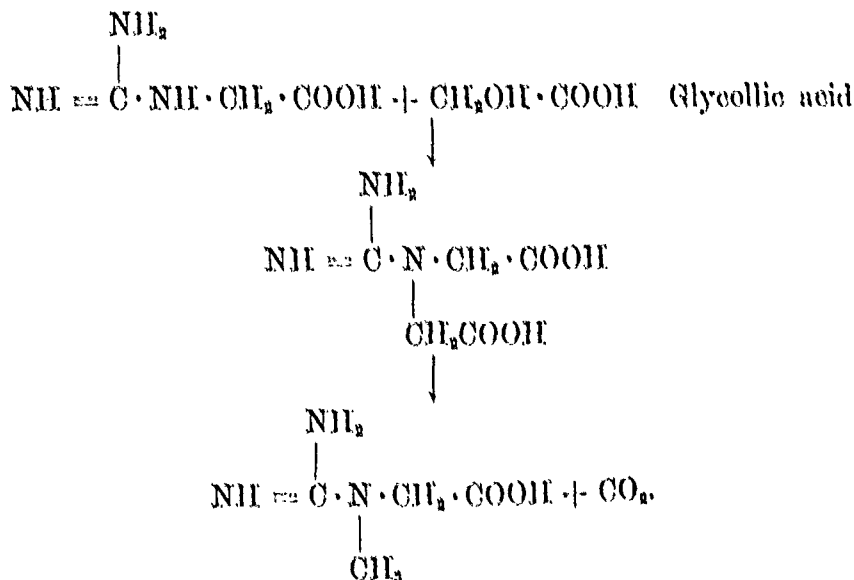
GUNNAR STEENSHOLT.

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It has been shown in recent years, by the work of several biochemists, that guanidine acetic acid plays a very fundamental part in the biosynthesis of creatine. Living cells produce creatine by methylation of guanidine acetic acid, the methyl groups being furnished by suitable methyl donators, among which methionine has been shown to play an important, and in all probability the dominating, rôle.

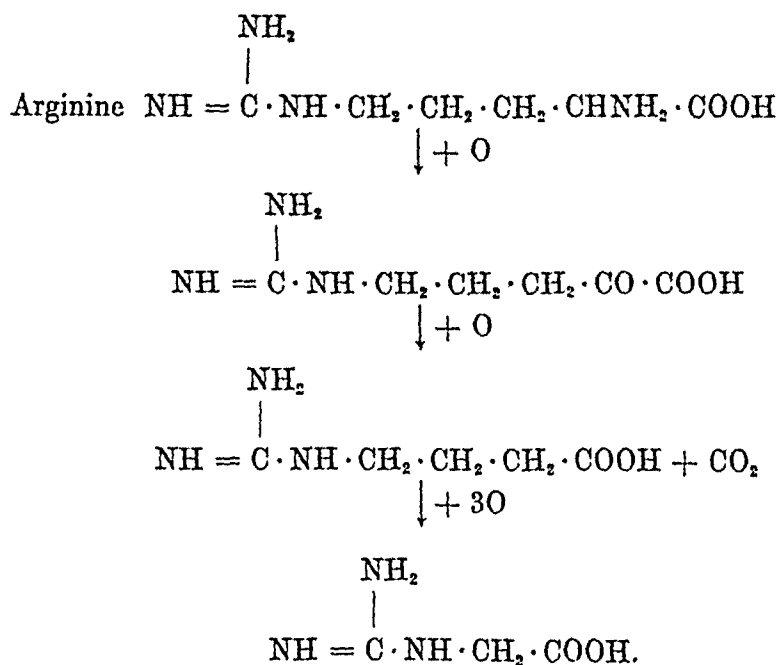
However, another very interesting possibility has been pointed out some time ago by DAVENPORT, FISCHER and WILHELM (1938), and supported by some experimental evidence. According to these authors glycollic acid takes part in the methylation of guanidine acetic acid in the way indicated by the scheme shown below:

Guanidine acetic acid

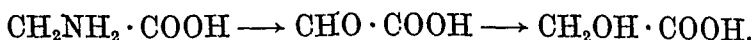


The authors carried out perfusion experiments on the isolated rabbit's heart, and found in the first instance that neither guanidine acetic acid nor glycollic acid alone would increase the total creatinine on being added to the perfusate in a concentration of 7 to 11 mg/100 ml. When both substances were added to the perfusate they found an increase in the total creatinine, and this increase was found to be due to an increase in the creatinine content of the heart plus perfusate. They further found that there is no significant disappearance of guanidine acetic acid from the perfusion system when it alone is present in the perfusate. In the presence of glycollic acid, however, an amount of guanidine acetic acid disappears which is approximately equivalent to the amount of creatine appearing. On the basis of these experiments, the details of which cannot be further discussed here, DAVENPORT, FISCHER and WILHELM I therefore concluded that guanidine acetic acid is converted quantitatively into creatine in the perfused heart in the presence of glycollic acid, and hence that glycollic acid acts as a methylating agent for guanidine acetic acid according to the above scheme.

We may finally mention that in order to account for the occurrence of guanidine acetic acid DAVENPORT, FISCHER and WILHELM I proposed the following chain of reactions:



Glycollic acid was supposed to be derived from glycine in the following way:



Though the ideas and results outlined above are mentioned by several writers on the subject, any more extensive attempt at a thorough verification by *in vitro* experiments on tissue slices or tissue extracts does not seem to have been undertaken so far. In a paper by BORSOOK and DUBNOFF (1940) it is mentioned that they have incubated rat liver with guanidine acetic and glycollic acid without finding any increase in the amount of creatine in the reaction mixture. Apart from this, verifications of the work of DAVENPORT, FISCHER and WILHELM, though obviously needed, do not seem to exist anywhere in the literature. In connection with some work of a related nature on biological methylation processes (STEENSHOLT (1945)) the present writer therefore thought it worth while to try to reinvestigate, by *in vitro* experiments, the problem of the action of glycollic acid as a methylating agent. The present note is a report on the results obtained.

Experimental Part.

Biological material. In all cases the biological material, whether liver, kidney, heart or muscle tissue, was removed from the freshly killed animal immediately after death, either in the laboratory or in the slaughter house. It was cut in pieces, placed on a watch glass and carefully minced by means of a pair of bent scissors. In this state it could be conveniently weighed and handled.

Substrates. The guanidine acetic acid was synthesised by the method of NENCKI and SIEBERT (1878). The glycollic acid was either a commercial preparation of the sodium salt, or two samples synthesised by the present writer by the procedures developed by FIRRIE and THOMSON (1880) and by HÖLZER (1883). The samples thus obtained were used simultaneously in all the experiments described below, and were found to behave in identically the same way in all cases.

Methods of analysis. The reaction mixtures were analysed for total creatinine by the FOLIN method, which is based on the picric acid reaction of JAFFE. We have applied the method in a form very similar to that developed by LIEB and ZACHERL (1934). The details of the procedure will be evident from the description of a typical experiment given below.

In order to check the results thus obtained analyses were also always carried out by the method of BENEDICT—BEHRE—LANGLEY—EVANS—LEHNARTZ (1936, 1941), in which 3,5-dinitro-benzoic acid is used as

a colorimetric reagent for creatinine. This method is believed to be of fairly high specificity, and was therefore regarded as a valuable check on the results obtained by the usual FOLIN method. The present writer has previously had occasion to apply the dinitro-benzoate method in a study of the methylation of guanidine acetic acid to creatine with methionine as a methyl donator (STEENSHOLT 1946).

The first experiments were carried out on rat tissue, and we shall quote the following one as the prototype of the whole series.

Muscle tissue was removed from the hind legs of a decapitated animal and prepared as described above. In two glass vessels of suitable size were placed

- 0.3 g muscle tissue
- 4 ml phosphate buffer (pH 7.0)
- 8 mg guanidine acetic acid
- 50 mg sodium glycollate.

Two other vessels contained the same amounts of tissue, buffer solution and guanidine acetic acid. All four reaction vessels were incubated for 18 hours at 38° C. After this period 4 ml of 20 % trichloroacetic acid were added to each vessel for deproteinization, and the precipitate removed by centrifugation. 2 ml of the deproteinized reaction mixture were pipetted into a 10 ml measuring flask. 1 ml n-hydrochloric acid was added and the mixture autoclaved for 20 minutes at 130° C. After cooling 0.4 ml of 10 % NaOH and 4 ml saturated picric acid were added, and the mixture left standing for 10 minutes. Water was then added up to the mark, and the flasks were ready for the colorimetric measurement.

The above method is essentially that of LIEB and ZACHERL already referred to. It was found that glycollic acid does not interfere with the creatine determinations by this procedure.

When compared colorimetrically no differences whatever could be observed between the four reaction mixtures. The mixtures with glycollic acid proved, on colorimetric examination to be exactly identical to those without the acid.

The experimental conditions were then varied in several ways. We used 4, 12 and 16 mg guanidine acetic acid instead of the 8 mg in the experiment just reported, and each test was carried through with incubation periods of 6, 12 and 18 hours. Each of these experiments was again varied by using respectively 25, 50 and 100 mg of glycollic acid. In all cases the results were invariably the same: when compared colorimetrically no differences could be found between the four reaction mixtures. Finally all these

experiments were repeated with McIlvaine's phosphatecitrate buffer replacing the ordinary phosphate buffer, and each experiment was carried out at pH 6.6 and pH 8. The results remained qualitatively unchanged; no trace of a creatine synthesis could be found.

Each of the experiments just described was checked by a duplicate experiment in which the determination of creatine was carried out according to the method developed by BENEDICT—BEHRE—LANGLEY—EVANS—LEHNARTZ already referred to. This was done in the following way. Four reaction mixtures were prepared and treated as above. At the end of the incubation period 4 ml 20 % trichloroacetic acid were added together with 1 ml 10 % hydrochloric acid. The mixture was centrifuged after 1 hour, and 5 ml of the supernatant liquid were autoclaved at 130° C for 30 minutes. After autoclaving the liquid was allowed to cool down to room temperature. Some methyl red indicator was added and the mixture was neutralised with 2.5 n NaOH. Water was added to make the total volume 11 ml. Now were added: 10 ml of a 6 % aqueous solution of the sodium salt of 3,5-dinitro benzoic acid, 10 ml of a 20 % aqueous solution of sodium acetate and finally 1 ml of a 2.5 n aqueous solution of sodium hydroxide. After shaking the flasks were left standing for 5 minutes. The contents were then quantitatively transferred to 50 ml flasks, which were subsequently filled up to the mark with water.

In separate experiments it was found that glycollic acid does not interfere with the determination of creatine by this method, at any rate not in the concentrations used in the present piece of work.

On subjecting the four reaction mixtures to colorimetric examination by the method just described, no differences whatever could be found between those with and those without glycollic acid.

All these experiments were carried out systematically with liver, kidney and heart muscle tissue from rats. There was never any increase in creatine content in the mixtures containing glycollic acid compared to those without the acid. The same program of work was then extended to other animals, and, all in all, muscle, liver, heart muscle and kidney tissue from rat, rabbit, cow, horse and pig have been examined. The results were in all cases the same as those quoted above for rat liver.

The discrepancy between our results and those of DAVENPORT,

FISCHER and WILHELM is obvious. The reason for this is, however, not easy to see. It should be remembered that for rat liver tissue our results agree with those of BORSOOK and DUBNOFF (1940). As regards our methods of analysis, their reliability appears to be well established, and they have previously been successfully applied to problems of biological methylation. To the present writer's mind, therefore, it appears probable, on the evidence given above, that glycollic acid plays only a minor rôle, if any at all, in the methylation process in question.

We may further mention that in view of what has been said in the introduction it is of particular interest to investigate the effect of substances like glycine on incubation with glycoeyamine and tissue suspensions. All in all, the above experiments on muscle, liver, heart muscle and kidney tissue from rat, rabbit, cow, horse and pig were repeated with the following substances replacing glycollic acid: glycine, serine, choline, betaine, ethanol amine, dimethyl ethanol amine, acetyl choline. The results were invariably the same; in no case could any formation of creatine be detected. For rat liver these results again agree with those of BORSOOK and DUBNOFF (*loc. cit.*).

It is interesting to note that BACH (1939) on incubating a suspension of chopped rat heart tissue with glycine and glycoeyamine observed a small increase in the content of creatine in his reaction mixtures. He states, however, that the effect is too small to justify any definite conclusions. It is further of great interest to note that RATNER, NOCITO and GREEN (1944) have recently discovered an enzyme in the liver, capable of catalyzing the oxidation of glycine to glyoxylic acid and ammonia. In view of the results presented in the present note it may be somewhat difficult to tell what bearing, if any, this discovery may have on the problem of biological methylation.

It has been shown above, probably in a fairly convincing manner, that a considerable discrepancy exists between the perfusion experiments and the later *in vitro* investigations. Hence it will presumably be agreed that it is not unimportant to attempt to clear up the reasons for this divergence by repeating and checking, if possible, the perfusion experiments, and some work along this line has been carried out by the present writer. Though our material is admittedly not so extensive as that of DAVENPORT, FISCHER and WILHELM (*loc. cit.*), it may nevertheless be of interest to give a brief report on the results obtained.

The perfusion experiments were carried out on the excised hearts of adult rabbits of body weight above 2 kg. The technique of heart perfusions experiments has been described a very great number of times both in periodicals and in text books, and any further description of technical details is therefore omitted here. The heart was perfused through the aorta in the familiar way, the perfusion liquid being ordinary, well oxygenated Ringer-Locke solution. Before the main experiment the perfusion fluid was allowed to pass through freely for some minutes to ensure removal of the blood. The perfusion experiment itself lasted from 75 to 100 minutes. After the experiment the heart and the perfusion fluid were removed from the apparatus. The heart was carefully minced, and extracted with trichloroacetic acid. An aliquot part of the filtered extract and the perfusate was analyzed for creatine by the method of BENEDICT—BEHRE—LANGLEY—EVANS—LEHN—ARTZ. This method was chosen on account of its fairly high specificity. Glycocyamine and glycollic acid were added to the perfusate in quantities of 10 mg per 100 ml liquid (in agreement with DAVENPORT, FISCHER and WILHELMI (*loc. cit.*)).

A difficulty with this method is that the perfused heart cannot be used again for control experiments. Controls can, however, be obtained by determining the amount of creatine in the hearts of other animals. The evidence obtained is therefore of a statistical nature, but this difficulty seems to be inherent in the nature of the problem, at least with the analytical methods at present at our disposal.

All in all 12 perfusion experiments were carried out, and 12 hearts were analyzed in order to serve as controls. The results are summarized in Table 1, which gives the weight of the hearts and the contents of creatine in the hearts, or in the hearts plus perfusate.

Table 1.

Controls		Perfused hearts	
Weight (in g)	Creatine (in mg)	Weight (in g)	Creatine (in mg)
6.0	10.8	6.2	10.5
6.5	12.0	6.8	11.0
7.1	12.9	6.9	12.0
6.1	11.2	7.1	10.0
6.8	11.5	7.2	12.6
6.5	11.5	6.0	11.1
6.0	11.0	6.2	11.0
6.1	10.5	7.0	12.2
6.3	12.0	6.3	11.5
7.0	12.8	7.0	12.6
6.6	12.0	6.1	10.5
6.8	11.8	7.0	12.0

An inspection of the table shows that there is no significant difference between the two groups. In both sets of measurements there is an average content of creatine of about 1.8 mg per gram of tissue, and the difference between the groups is too small to have any importance. Although such a close agreement may be in part fortuitous, it will probably be agreed that we are justified in considering the results of the perfusion experiments to be in good accord with those of the *in vitro* investigations reported above. There is no sign of any formation of creatine by either procedure.

The writer is glad to express his best thanks to Professor R. EGE for generous hospitality and support.

Summary.

A brief review is given of certain current ideas on the rôle of glycollic acid as a methylating agent. Next the results are reported of some *in vitro* experiments in which guanidine acetic acid and glycollic acid or glycine, and some other substances as well, were incubated with suspensions of various animal tissues. The determinations of creatine in the reaction mixtures were carried out both by the picric acid and by the dinitro-benzoate methods. The results were negative: in no case could any trace of a creatine synthesis be demonstrated. Finally some perfusion experiments on the excised rabbit's heart were carried out. The results were in substantial agreement with the *in vitro* experiments. We may therefore be justified in concluding that glycollic acid is incapable of methylating guanidine acetic acid to creatine. The relation of these results to some previous investigations by other workers is discussed.

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On an Amine Oxidase in Rabbit's Liver.

By

GUNNAR STEENSHOLT.

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The oxidation of amines in the animal organism is a process of great biological importance, and by the efforts of several workers a great amount of experimental material, bearing on the problem of enzymatic oxidation of amines, has been brought together. A satisfactory interpretation of a large part of the experimental findings has been achieved by assuming the presence in the animal body of two main amine oxidizing enzymes, the mono amine oxidase and the diamine oxidase. The first of these attacks substrates with one amino group, like butyl amine, amyl amine and iso-amyl amine, and also amines containing aromatic rings, like tyramine, adrenaline, tryptamine and β -phenyl ethyl amine. The diamine oxidase catalyzes the oxidation of amines with two amino groups, like histamine, cadaverine and putrescine. By inhibition experiments these two enzymes have been proved to be chemically different.

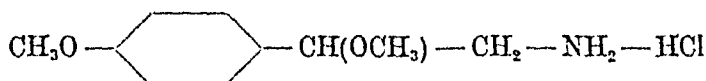
It seems, however, that other enzymes capable of oxidizing various amines occur in Nature. We exclude from consideration such enzymes as the amino acid oxidases, and direct attention to the following observations. It was shown by BLASCHKO, RICHTER and SCHLOSSMANN (1937) that mescaline is only very slowly oxidized by rat liver. BERNHEIM and BERNHEIM (1938), however, found that rabbits' liver is capable of rapidly oxidizing this substrate. Mono amine oxidase preparations from rabbit's liver were found to exhibit the following properties: Mescaline is oxidized to the corresponding acid, which has been isolated (see also SLOTTA and MÜLLER (1936)). The theoretical amount of ammonia is recovered. Unlike the oxidation of tyramine, the oxidation of mescaline is inhibited by pyrophosphate, borate and relatively high concentrations of cyanide. As is the case with

tyramine the rate of oxidation of mescaline is increased by increasing the oxygen tension.

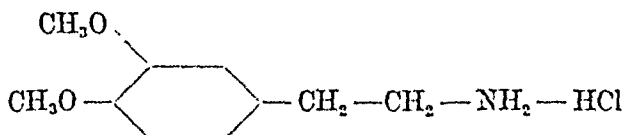
From their experiments the BERNHEIMS conclude that some other factor than the mono amine oxidase itself is necessary for the oxidation of mescaline. Since pyrophosphate, borate and cyanide are well known to form complexes with heavy metals, they suggest that some heavy metal may be necessary together with the mono amine oxidase, to effect the oxidation of mescaline, the metal thereby acting as a kind of coenzyme. The possibility that an entirely new enzyme may be at work does not seem to have been adequately discussed by the aforementioned authors, and the problem does not seem to have been treated elsewhere in the literature except for a very brief mention by ELLIOTT (1940). It is the purpose of the present note to report on some experiments which seem to have a bearing on the nature of the enzyme catalyzing the oxidation of mescaline and similar substances.

Experimental Results and their Discussion.

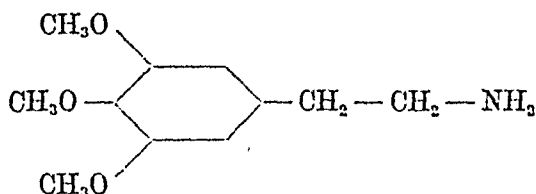
Materials and methods. The following substrates were used:



β -(-4-methoxy-phenyl)- β -methoxy-ethyl amine hydrochloride, for convenience referred to simply as substance A.



β -(-3,4-dimethoxy-phenyl)-ethyl amine hydrochloride, referred to as substance B.



β -(-3,4,5-trimethoxy-phenyl)-ethyl amine, or mescaline. It was used either as the hydrochloride or the hydrosulphate.

These substances were obtained from F. Hoffmann-la Roche & Co. to whom my best thanks are due for their generosity.

A sample of substance B was also synthesized by the writer. This was carried out in the following steps. Firstly, 3,4-dimethoxybenzaldehyde (veratrum aldehyde) was obtained from vanilline and dimethyl sulphate either in the presence of potassium hydroxide or in the presence of sodium hydroxide in methyl alcoholic solution. By heating with anhydrous sodium acetate and acetic anhydride the veratrum aldehyde was converted into 3,4-dimethoxy cinnamic acid. By treating this acid with sodium amalgam in alcoholic solution 3,4-dimethoxy-hydro-cinnamic acid was obtained. The amide of this acid was then obtained via the acid chloride, which in its turn was obtained in the well known way by treating the acid with phosphorus pentachloride. As the final step the acid amide was treated with aqueous hypobromite, and substance B was subsequently isolated from the reaction mixture. The technical details of all these procedures cannot be given here (see VON KOSTANECKI and TAMBOR (1906, PERKINS and SCHIESS (1904), PERKINS and ROBINSON (1907), PICTET and FINKELSTEIN (1909)).

All measurements of oxygen uptake were carried out in the Warburg apparatus in the usual manner; sufficient details will be given below. Duplicate experiments were of course always carried out. The temperature in the water bath was 37° C.

The source of the enzyme preparations used in this work was the livers of rabbits and rats. The organs were removed from the animals immediately after death. Active enzyme preparations were obtained in the following manner. The livers were placed on a watch glass and finely divided with a pair of bent scissors. The homogeneous mass thus obtained was thoroughly ground with sand in a mortar and then extracted with 0.05 M phosphate buffer at pH 7.5 to 7.8 (about 1 ml of buffer solution per gram of tissue), and finally pressed through muslin. The enzyme solution thus obtained was used in many experiments. A further preparation was made by dialyzing the extract just described against distilled water until the solution showed no or only a very small oxygen uptake in the Warburg apparatus. Many experiments were also carried out directly with the liver pulp itself. This is of course the crudest possible enzyme preparation, but it is nevertheless useful and convenient for many purposes.

It was first of all verified that mescaline is only very slowly oxidized by the enzyme solutions obtained from rat liver, or by rat liver pulp, while the corresponding preparations from rabbit's liver had a strongly oxidizing effect. We further verified that cyanide, borate and pyrophosphate inhibited the oxidation of mescaline in concentrations at which the oxidation of tyramine and iso-amyl amine was not affected at all. It was also confirmed that cyanide does not inhibit the action of mono amine oxidase.

We next investigated whether substrates A and B could be oxidized by our liver preparations. It was found that substrate A gave rise to no oxygen uptake at all when working with either

rat liver or rabbit's liver. Hence this compound is not attacked by the enzymes in question.

To substantiate this remark we shall quote as an example the following experiment, chosen at random from our laboratory notes. It was carried out liver pulp as enzyme preparation. The main chamber of a Warburg vessel contained 2 ml phosphate buffer solution (pH 7.4). The central chamber contained 0.2 ml 5 per cent potassium hydroxide solution for the absorption of carbon dioxide. The side chamber contained 0.2 ml of a solution, prepared by dissolving 5 mg of substance A in 1 ml water. A suitable amount of liver pulp was placed in the main chamber. A thermobarometer was, as in all our experiments, arranged in the familiar way. After equilibration in the water bath for 15 minutes the stopcocks were closed, and the oxygen uptake followed for a period of 30 minutes. The amine solution in the side chamber was then tipped into the main chamber, and the measurements continued for another 30 minutes period. No difference in the oxygen uptake during two periods of observation could be found.

Work with liver pulp from the rabbit gave similar results, as did also work with the enzyme solutions described above, both the undialyzed and the dialyzed one.

Our experiments with substance B, however, gave a different result. With rat liver preparations, whether enzyme solutions or liver pulp, there was practically no extra oxygen uptake to be observed, which could be ascribed to an oxidation of the substrate. With preparations from rabbit's liver, however, a considerable oxidation was found to take place. Thus, when liver pulp was used and the Warburg vessels were prepared as just described, we found in the second half hour period an increase in oxygen uptake of 30, 71, 50 and 52 per cent in four successive experiments. Confirmatory evidence was of course obtained with the enzyme solutions, both the undialyzed and the dialyzed ones. The effect is therefore beyond question. The relative rates of oxidation of mescaline and of substance B were determined by comparing the oxygen uptakes when equal quantities of the substrates were oxidized by equal amounts of the two enzyme solutions. Thus, with four different undialyzed solutions we found for the ratio

$$\frac{\text{rate of oxidation of mescaline}}{\text{rate of oxidation of substance B}}$$

the numerical values 1.7, 1.6, 2.1 and 2.2. With two different dialyzed solutions we found the values 1.8 and 2.2.

Some inhibition experiments with substance B were then carried out. By the Warburg method we investigated the effect of cyanide, borate and pyrophosphate on the oxidation of the compound, and found results in substantial agreement with those obtained for mescaline by the BERNHEIMS and later by ourselves. Thus, 0.0001 M potassium cyanide was found to have no perceptible effect on the process. 0.001 M cyanide gave a ca. 30 per cent inhibition, while 0.007 M sodium borate and 0.09 M potassium pyrophosphate both gave about 60 per cent inhibition. These data were obtained for the oxidation of 2 mg of substance B at pH 7.7. An almost 90 per cent inhibition was obtained with 0.006 M potassium cyanide.

It has been found by the workers mentioned in the introduction that mono amine oxidase is inhibited by ethyl urethane, and this was verified by the present writer for tyramine and β -phenylethyl amine. Mescaline and substance B, however, showed a different behaviour. A typical experiment was carried out as follows. One Warburg vessel contained in its main chamber 2 ml of an undialyzed enzyme solution (pH 7.5) and 2 mg ethyl urethane dissolved in 1 ml water; the main chamber of another vessel contained 2 ml of the same enzyme extract plus 1 ml water. Both chambers contained in addition 2 mg mescaline and in a parallel experiment 2 mg of substance B. The central chambers contained 0.2 ml 5 per cent potassium hydroxide solution, as usual. No difference in oxygen uptake was found, and similar results were found when working at other urethane concentrations (up to 8 mg ethyl urethane). Similar results were also found for the dialyzed enzyme extracts.

It has been found by previous workers that ephedrine is not attacked by mono amine oxidase. However, this compound has a very strong affinity for the enzyme, and is therefore capable of competitive inhibition of the oxidation of other amines by mono amine oxidase. This was verified by the writer in some work with tyramine and a mono amine oxidase preparation from rat liver. It was thought to be of interest to see whether ephedrine has any effect on the oxidation of mescaline and substance B by the enzyme preparations from rabbit's liver. The experiments were carried out by a procedure very similar to that used for ethyl urethane, and details are therefore unnecessary. No effect was found.

We have finally investigated the effect of some metal salts on

the oxidation of mescaline and substance B. The salts investigated were the nitrates and chlorides of copper, iron, manganese and magnesium. The experiments were carried out as described above for ethyl urethane and ephedrine. The main chamber of the Warburg vessels contained 2 mg mescaline or substance B, and both dialyzed and undialyzed enzyme extracts were used. The effect of each salt was investigated with quantities of 0.1, 1.0, 1.5 and 2 mg of it being present in the reaction mixtures. There was never any sign of an activating effect of the salts in the oxidation process.

Throughout all our work mescaline hydrochloride and mescaline hydrosulphate showed identical behaviour.

Discussion. From the above experiments with various inhibitors we may probably safely conclude that we are dealing with an essentially new enzyme, certainly different from the mono amine oxidase with respect to chemical constitution. This is borne out very clearly for instance by the inhibition experiments with ephedrine. Our work with metal salts indicates that in all probability copper, iron, manganese and magnesium are not required by the enzyme. It should also be remembered that the cyanide inhibition observed for the mescaline oxidase by the BERNHEIMS as well as by the present writer is not that of a true cyanide sensitive system, which should be inhibited almost completely with a concentration of 0.001 M potassium cyanide, and this was not the case in our experiments. In view of all this it appears rather improbable that the mescaline oxidizing enzyme should be simply mono amine oxidase plus some heavy metal as coenzyme.

From our experiments it seems as if the presence of at least two methoxy groups in the aromatic rings in the substrates is necessary for the action of the enzyme, and it might be tempting to speculate on the rôle of these groups for the affinity between the enzyme and its substrates. Actually, however, more work with many different substrates is required to provide the experimental basis for work along that line, and the problem therefore has to be reserved for a later occasion.

A word may finally be said about the purification of the enzyme, which seems to present a rather difficult problem. We did not succeed in separating the mescaline oxidase from the accompanying mono amine oxidase. Thus autolysis and heating to different temperatures proved ineffective for this purpose. We

further tried adsorption on Fuller's earth, some aluminium oxide preparations, kieselguhr and a few other adsorbents available in our laboratory, among them two types of permutite. No separation was achieved. We mention this only to show that there is a considerable similarity in many respects between mescaline oxidase and mono amine oxidase.

The writer is glad to express his best thanks to Professor R. EGGE for hospitality and support.

Summary.

The paper reports the results of an investigation of the enzyme in rabbit's liver that catalyzes the oxidation of mescaline. Another substrate for this enzyme is found. By inhibition experiments it is shown that the enzyme is in all probability structurally different from the mono amine oxidase. The experiments indicate that copper, iron, manganese and magnesium are probably of no importance for the enzymatic process. The rôle of metals is further discussed, and it is shown that a suggestion about the nature of the mescaline oxidizing enzyme put forward by BERNHEIM and BERNHEIM is probably untenable.

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The Elicitation of Viscero-motor Reflexes.

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Ever since LENNANDER (1902) showed, that mechanical stimulation of the abdominal viscera did not evoke pain sensation under local anesthesia, the question of sensitive innervation of the viscera has been an object of a special interest. Investigations of this problem have included observations on humans, inter alia during operation, as well as experiments on animals, whereby certain typical reactions and reflexes to "pain" stimuli have been studied. During the latter type of investigation observations could be made particularly of the production and transmission of the nerve impulses, which might be considered to be the basis of pain sensations.

Previous investigations of the visceral supply of end-organs for centripetal impulse discharge have however not led to any generally accepted opinion. LENNANDER's opinion is still maintained that viscera are lacking in true pain nerves and that the occurrence of visceral pain is produced by simultaneous irritation of the pain sensitive parietal peritoneum or mesentery. (MORELEY, 1931.) The phenomenon, that mechanical stimuli of the viscera do not evoke pain whereas viscera can be the site of intensive discomfort under pathological conditions, has been explained also in an other way, namely, that the visceral pain end-organs are adapted for specific types of stimuli, such as, pulling, stretching and anoxia and are only very slightly sensitive to other forms of stimuli. Such an explanation is difficult to accept, however, since

mechanical stimulation, *e. g.* incision in the organ, or injury to the tissues due to a severe pressure must be considered to evoke a direct stimulation of the pain nerve fibres, which must produce the same sensation as stimulation of the end-organs.

LEWIS and KELLGREN (1939), during experiments on the elicitation of visceromotor reflexes in decapitated cats, have claimed to have shown that nerve fibres for pain transmission and their end-organs are located only in the mesentery and are entirely lacking in the intestine as well as in the solid organs of the abdomen. These authors were thus able to evoke visceromotor reflex through mechanical stimulation of the mesentery and the pancreas localized therein but not through stimulation of the intestine. The mesentery and its end-organs may be simultaneously stimulated by a stretching or pulling of an intestinal section and in this way a pain sensation may be evoked. In contrast, however, to the results found by LEWIS and KELLGREN, there are the statements of MILLER and his associates (1925) who found that squeezing the small intestine of a decapitated cat elicited contractions, *inter alia*, in the abdominal muscles. DOWNMAN and Mc SWINEY (1946) showed on decerebrated cats, where the spinal cord had been severed in the upper thoracic part, that increased tone in the skeletal muscles may be elicited by stimulation of the small intestine without involving the mesentery.

GERNANDT and ZOTTERMAN (1946) recorded action potentials in nerve branches in the mesentery and in the splanchnic nerve using strong mechanical stimulus of the small intestine and found impulse discharges in the δ and C fibres, *i. e.* the same types of fibres that in the cutaneous nerves transmit pain and nociceptive reflexes (ZOTTERMAN 1933, 1936, 1939). Partly on the basis of the results presented in this paper GERNANDT and ZOTTERMAN assumed that the recorded action potentials originate from afferent "pain" conducting fibres.

In the present work experiments are related, which seem to elucidate the question of the elicitation of the visceromotor reflexes especially by stimulation of the intestine.

Method.

The experiments were made on cats, some under superficial Dial anesthesia and some after decapitation, the latter animals being ventilated with the help of a Starling pump. At the beginning of the

experiments the animals were generally in good condition with active spinal reflexes. Usually it was possible to begin the experiments 20—30 min. after decapitation and no signs of spinal shock were than observable. That the usually stated shock stage following decapitation could be shortened is probably to be attributed to our attempt to make the decapitation as quickly as possible using the least possible amount of ether. In some cases the spinal automatism has been so pronounced that it made the recording difficult. Even under Dial anesthesia the spinal reflexes have been well preserved during the experiments.

In solitary experiments rabbits have been used under Dial anesthesia and after decapitation.

Contractions of the partially freed segment of the m. rectus abdominis have been registered by a mechanical myograph as well as by electromyography. Mostly the mechano-myogram has been registered isotonically using a lightly weighted writing lever. Registration of the action potentials has been made with the help of concentric pin electrodes and a direct-alternate current amplifier, type Buchthal-Nielsen, connected to a mirror oscillograph, type Electrical-Phonofilm. During these experiments the animal has been placed in a screened box with a temperature of 30—38° C (saturated humidity).

For experiments on the stimulation of the small intestine, an intestinal loop has been pulled out through a small opening in the abdominal wall, lateral and on opposite side to the freed segment of the rectus muscle.

An electron stimulator, type Buchthal-Kaiser, and silver pin electrodes have been used for electrical stimulation.

Results.

A. Mechanical Stimulation.

The freed loop of the small intestine has, *inter alia*, been stimulated by squeezing with an artery forceps, naturally, in this way, not only a pressure affect but even tissue injury has been obtained. By direct registration of muscle activity as well as by recording the action potentials from the rectus muscle (see fig. 1 and 2) absolutely clear contractions could be demonstrated both on decapitated and on Dial narcotised cats. By application of the artery forceps over the entire transverse section of the intestinal loop greater contractions were obtained than if only half of the section was stimulated, provided the mesentery was not simultaneously irritated (fig. 2). Every pulling of the mesentery was carefully avoided.

With similar stimulation of the stomach wall, mesentery, large intestine and kidney, reflex contractions were obtained which varied in degree for the point of stimulation.

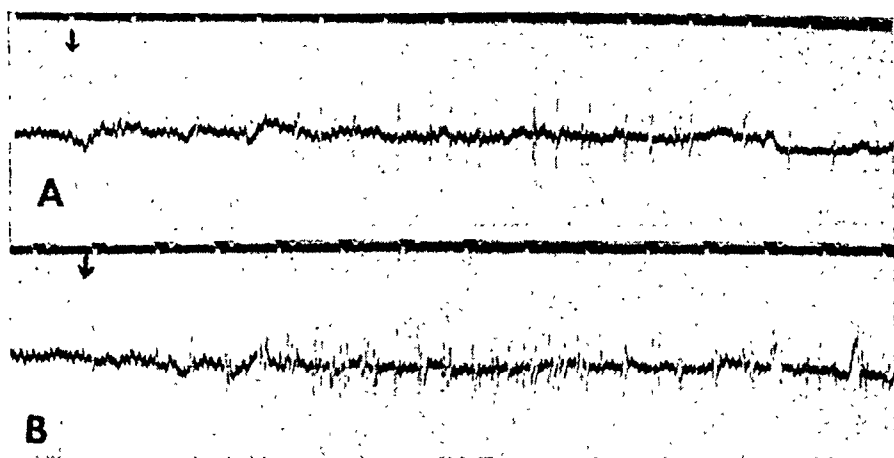


Fig. 1. Cat, decapitated. Electromyogram from m. rectus abdominis. A. Squeezing of the small intestine with artery forceps. B. Pulling of the mesentery. Time $\frac{1}{5}$ sec.

Similar stimulation of an equally large skin area, freed from the abdominal wall, gave always a much stronger contraction than that from the small intestine (fig. 3 A and B).

The abdominal wall, freed from skin, on the opposite side from which registration was made, also gave a stronger muscle contraction with similar stimulation than that from the intestine but less than that from the skin.

The myogram for these stimuli showed a quick phase of contraction and a slow relaxation.

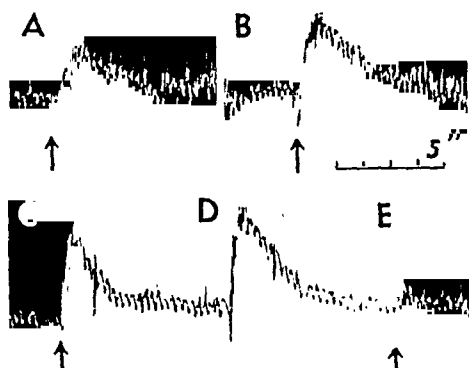


Fig. 2. Cat, decapitated. Myogram from m. rect. abd. Forceps on small intestine A. longitudinally on the side opposite the mesentery insertion. B. The same nearer the mesenterial connection. C. Transversely over half the intestinal section. D. The same, over the whole section. Time in 5 sec.

traction and a slow relaxation. The contraction curves were of the same general shape by stimulation of the intestine, skin or other organs. A summation effect was obtained with simultaneous stimulation of different areas; while subsequent stimulation of different areas gave an addition effect.

In order to obtain a longitudinal pulling stimulation of an intestinal loop, 2 artery forceps were placed 2—4 cm. apart, after the effect of this latter irritation entirely sub-

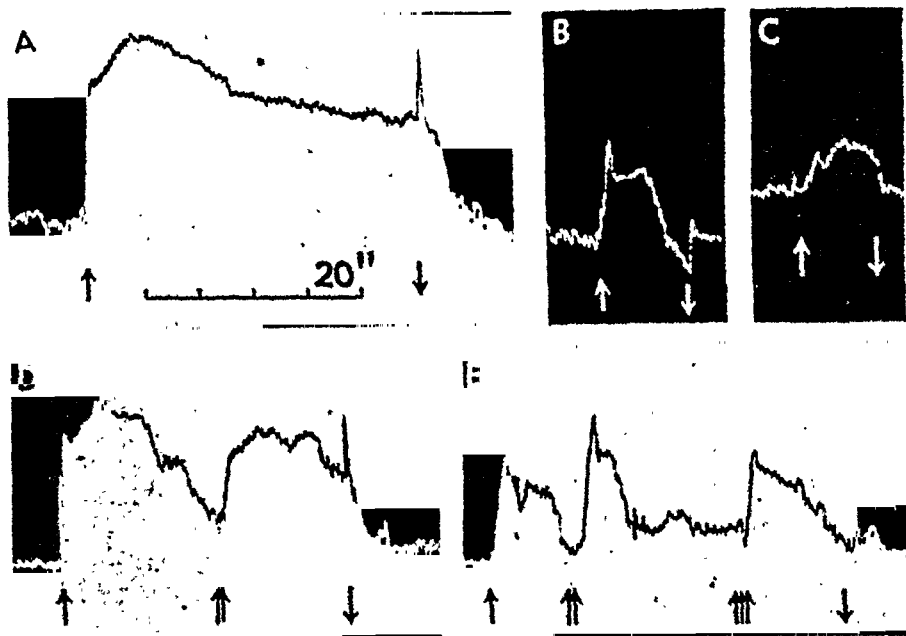


Fig. 3. Cat, decapitated. Myogram from section of m. rect. abd. A. Squeezing of freed abdominal skin with forceps. B. The same, of the small intestine. C. Stretching with balloon of small intestine, cir. 4 cm. long area. D. ↑ pulling in the mesentery, ↑↑ further pulling. E. ↑ forceps on the small intestine, ↑↑ another forceps cir. 4 cm. caudal. ↑↑↑ pulling between the two forceps. Time in 20 sec.

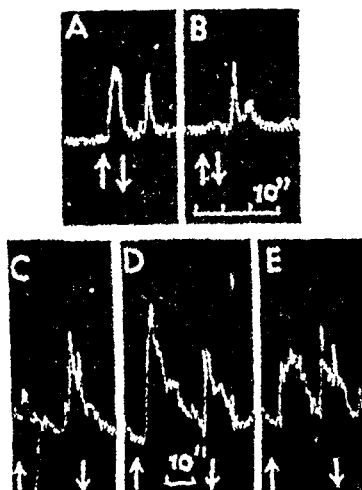


Fig. 4. Cat, decapitated. Myogram from m. rect. abd. Electrical stimulation of the small intestine. A. Near mesenteric connection, short duration; B. As distal to mesenteric connection as possible, short duration. C. Position as (B), long duration. D. Position as (A), long duration. E. Position between (A) and (B), long duration. The first contraction wave varies with point of application, the other varies with duration. Time in 10 sec.

sided, pulling was effected between the two forceps. Even here a contraction was evoked in the rectus muscles, which within certain limits increased with increased tension. A smaller contraction was obtained, however, with a very strong pull on a

4 cm. long intestinal segment than that from the application of either of the two forceps (fig. 3 E).

Also stretching of an intestinal loop has been obtained by insertion a small rubber bladder, which could be inflated to varying degrees through a hose connection. The bladder was generally inserted in the middle section of the small intestine. Moderate and intensive stretching in this way of a 4 cm. long section elicited a muscle contraction very nearly similar in strength to that obtained by pulling an equal area (fig. 3 C). The effect of extensive stretching obtained in both these ways has thus not been more pronounced than that obtained by pressure of forceps on a much smaller area. Therefore, it has been demonstrated that it is easier to evoke visceromotor reflexes by injury of the intestinal wall than by pulling and stretching.

On rabbits under Dial anesthesia or after decapitation a visceromotor reflex could never be evoked except from branches of the splanchnic nerve and by mechanical stimulation of the hilus area of the kidney.

B. Electrical Stimulation.

The electrode distance was 4 mm. for electrical stimulation of the freed intestine. A contraction of the rectus muscle was obtained in direct connection to the stimulation. The nearer the electrodes were placed to the point of connection between the intestine and the mesentery the greater was the reflex contraction (fig. 4 A B).

A relatively slowly appearing contraction of the stimulated area was evoked when electrical stimulus of a few seconds duration was applied to the intestine. This local intestinal contraction appeared as a marked constriction and decided paling. In connection with the appearance of the intestinal contraction a reflex contraction was evoked in the rectus muscles. Thus two waves were obtained on the myogram with stimuli of favorable duration, the first had much the same appearance as that from mechanical stimuli — a quick rise and a slower sinking towards the baseline — the second, usually with a slower rise in association with the appearance of intestinal contraction (fig. 4). The latter could, however, attain and even surpass the maximum of the former, due partly to localisation of the stimulus with respect to the mesenteric connection — although this had particular influence on the size of the first wave — and partly on the duration of the stim-

ulus — which particularly influenced the intestinal contraction and thereby the size of the second wave. The difference in size between the two waves of contraction, evoked by electrical stimulus of the intestine, was however not greater than that it can be explained by the intestinal contraction involving a larger area than that which was directly affected by the electrical stimulation.

Experiments with electrical stimulation have thus shown:

1. that a visceromotor reflex can be elicited by electrical stimulation of a limited area of the intestine even on the side opposite the mesentery.
2. that a larger contraction is obtained the nearer the stimulated area is to the mesenteric connection.
3. that a local intestinal contraction, caused by electrical stimulation, can elicit a visceromotor reflex.
4. that quite strong reflexes can be elicited as well by pure electrical stimulation of a part of the intestine as by contraction of the same.

C. Chemical Stimulation.

1/10 N HCl and BaCl₂ solutions of varying concentrations have been used as stimuli.

Regular contractions of the rectus muscle have been obtained by dropping 1/10 N HCl on the intestine, regardless of whether an intestinal contraction occurred or not.

Abdominal muscle contractions associated with local intestinal contractions occurred by dropping BaCl₂ solution on the intestine in such low concentrations that when injected in the skin no motor reflexes were elicited. This indicates that the effect was produced by the intestinal contraction evoked by this agent and not directly by the BaCl₂ solution.

D. Thermal Stimulation.

Abdominal muscle contractions were evoked if hot Ringer solution was dropped on the intestine, even when care was taken not to stimulate the mesentery. Local intestinal contractions were not elicited in this way, therefore it must be assumed that the afferent nerve fibres in the intestinal wall, or their end-organs, were thermally stimulated.

Discussion.

The elicitation of visceromotor reflexes from the intestinal wall in the above experiments shows that the wall is supplied with afferent conducting nerves. This confirms the investigations of DOWNMAN and Mc SWINEY (1946). It, however, contradicts the published results of LEWIS and KELLGREN (1939), which led them to the hypothesis that pain stimulation only should be able to be elicited by pulling the mesenterial connection of the intestine. Since we used the same method as LEWIS and KELLGREN, we cannot explain the difference in results other than that these authors used animals which were in a less irritable condition. Since all the nerve fibres to the intestine run in the mesentery the density of nerve fibres there must be many times greater than in the intestinal wall. As the effect of a stimulus is dependent on the total number of nerve fibres involved it is to be expected that it is easier to obtain clear effects by stimulating the mesentery than only a part of the intestinal wall. Thus in our experiments on animals with reduced spinal reflex irritability, registerable effects could only be elicited by stimulation of the mesentery, while on the other hand, clear effects have been obtained by stimulation of the intestinal wall of animals with good spinal irritability.

The visceromotor reflexes in our experiments have been elicited by mechanical, electrical, chemical and thermal stimuli. In our comparisons between the effects of stretching and pulling of the intestine and other forms of stimuli, we could not find any support for the theory that the afferent nerve fibres with end-organs, under discussion here, should be more easily stimulated by the former type of stimuli than the latter. We have, therefore, not been able to confirm the opinion that nociceptive reflexes and the possibly associated pain sensations should be preferably elicited by stretching and pulling the intestinal wall. Quite the contrary, our experiments have shown that the different types of stimuli, which elicit pain sensation and nociceptive reflexes from the skin and other somatic tissues even elicit the visceromotor reflex.

The experiments have thus shown that the intestinal walls in the cat are supplied with nerve fibres and end-organs which upon noxious stimulation transmit nociceptive reflexes and thereby react as pain transmitting elements in somatic innervated tissues.

This has been confirmed by the investigations of GERNANDT and ZOTTERMAN (1946) concerning visceral pain, already referred to.

It is however not altogether certain that a stimulus, which elicits a motor reflex also evokes pain sensations. It is possible that a higher degree of spatial and temporal summation is necessary in order to evoke pain sensation than that necessary to elicit a visceromotor reflex. This in connection with the low nerve fibre density in the viscera could explain their relative insensitivity to stimuli of short duration. With stimuli of longer duration, the necessary conditions exist for the occurrence of a pain sensation, which then is usually associated with other indications of summation, such as hyperalgesia in the dermatoms, belonging to the same segments as the organ in question.

Summary.

The elicitation of visceromotor reflex especially from the small intestine has been studied on the cat under light barbituric anesthesia or after decapitation.

On animals with good reflex irritability, the reflex can be elicited from the intestinal wall by mechanical, electrical, chemical and thermal stimuli without the involvement of the mesentery or of its connection with the intestine.

The effect of a stimulation of the intestinal wall is stronger the nearer the stimulus is placed to the mesenteric connection. This is explained by the fact that the density of the nerve fibres must be greater here.

A cutano-motor reflex is much stronger than a visceromotor reflex elicited by the same stimulus on an equal area of skin resp. intestinal wall.

No basis has appeared for the assumption that the intestinal wall should be supplied with pain nerve end-organs, especially adapted for stretching or pulling, or which in any way should differ from the pain nerve end-organs in the somatic innervated tissues. The principal difference between the visceral and somatic innervated organs probably is a quantitative, the density of nociceptive nerve fibres with end-organs.

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The Regulation of the Body-temperature during Work Performed with the Arms and with the Legs.

By

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M. NIELSEN (1938) has shown that the increase in body temperature during muscular work is due to a regulation and — inside wide limits — independent of the environmental temperature, humidity and air movement. The higher body temperature during work, therefore, is not due to an inadequate heat regulation, unable to cope with the increased formation of heat in the working muscles, but is due to a new "setting" of the thermo-regulatory centre. The usefulness of a higher body temperature during exercise has been shown by ASMUSSEN and BØJE (1945) to lie in the fact that the muscles perform better at a higher temperature.

The "setting" of the thermo-regulatory centre during work may be effected in various ways. NIELSEN suggests cortical impulses or changes in the composition of the blood, but is well aware that other factors, such as reflexes from different parts of the body, also may play a rôle. Since NIELSEN'S paper appeared, very little has been done in attempting to elucidate this question. An article by WINSLOW and GAGGE (1941) mentions — in affirmation of NIELSEN'S observations — that the skin temperature during work is rather independent of the intensity of work and that the rate of sweating, consequently, must be controlled by the internal temperature rather than by the skin temperature.

As a means of studying the changed sensitivity of the thermo-regulatory centre during work a comparison of the heat regulation during arm-work and work performed with the legs seemed possible. It is well known that for instance the adaptation of the ventilation to the oxygen consumption is different in arm-work and in leg-work [ASMUSSEN and NIELSEN (1946)] and it has been suggested that this difference might be due to both a dissimilarity in the composition of the blood and to an inequality of the reflexes arising in the arms and in the legs. It is, therefore, the purpose of this paper to study the increase in temperature during work performed with the arms and with the legs, and, if possible, from the results to draw such conclusions that might add to our knowledge of the thermo-regulation during work.

Methods and Procedure.

The work was performed on a KROGH-bicycle ergometer, which for the arm-work could be furnished with a pair of handles in stead of the pedals. During armwork the subject sat on a high stool.

The temperatures were measured by a thermo-couple inserted about 20 cm into the rectum. In some experiments the temperature in the stomach was measured by a second thermo-couple, placed within a small metal-bulb, about 4 mm in diameter, that could be swallowed easily. The wires were inclosed in a rubber tube. The reference temperature was measured with a mercury thermometer, placed in water in a DEWARS-flask, kept at 38–39° C. The thermometer could be read with an accuracy of 0.02° C. On the galvanometer (Multiflex), 1 scale unit corresponded to 0.103° C, so that also here the accuracy was about 0.02° C.

The total energy output in Cal/min was calculated from the oxygen uptake and the RQ, both determined by the DOUGLAS-bag method. The heat production then could be estimated after subtraction of the external work as measured on the ergometer.

The subjects were two young, male students, P. Th., age 21, and O. W. age 19. All experiments were made at room temperatures of 20–22° C under standard conditions in the morning and after a preliminary resting period in the lying position of at least half an hour. The work lasted in all cases 40 minutes. At this time a steady state was reached in works of lighter intensity, but in heavy work the temperature was still increasing. However, as the subjects were unable to continue heavy work with the arms long enough to reach a steady state, the temperature after 40 minutes was chosen as the working temperature at all grades of work.

As NIELSEN has pointed out, the temperature reached by a certain intensity of work is to some extent depending on the resting temperature previous to exercise. This, combined with the fact that the

absolute temperatures — especially in the experiments on subject P. Th. — were less accurately determined, make the *increase* in temperature more reliable than the *absolute* temperatures. In the present work, therefore, the increase in temperature and not the final temperature was taken as a measure of the influence of exercise on body temperature.

Results.

The results from a pair of typical experiments with light work on P.Th. (360 mkg/min with the arms, 540 mkg/min with the legs) are presented in fig. 1. In both kinds of work the energy output was 7.2 Cal/min. Due to the lower efficiency of the arm-muscles for this kind of work, the heat production was 6.4 Cal/min when working with the arms as compared to only 5.9 Cal/min when working with the legs. It will be seen, that the increase in temperature above the resting value is larger when working with the legs than during arm-work.

Fig. 2 shows a corresponding set of experiments on O. W. during heavy work (890 mkg/min with the arms, 1,214 mkg/min with the legs). The energy production was in both cases approximately 14.3 Cal/min and the heat production 11.5 Cal/min during leg-work and 12.2 during arm-work. Although no steady state of body temperature was reached, it is easy to see, that also here the increase during exercise with the legs is larger than during work with the arms.

A summary of all the experiments made on P. Th. is given by figs. 3 and 4, where the increase in temperature after 40 min of work is plotted against the total energy output per minutes (fig. 3) and the heat production per minute (fig. 4). It will be seen that in both figs. the increase is larger when work was performed with the legs than when it was done with the arms.

Corresponding results were obtained with O.W. In 20 experiments with leg-work and 19 with arm-work, the latter as heavy as the subject could perform, the mean results came out as in table 1.

As it might be objected that the higher increase in temperature during work with the legs were due to the proximity of the measuring thermo-couple to the working muscles in the legs or to the great veins coming from the legs, a series of experiments was made in which the temperature increase in the *stomach* was measured. The subject swallowed the ball containing the thermo-couple previous to work, but it was withdrawn immediately after

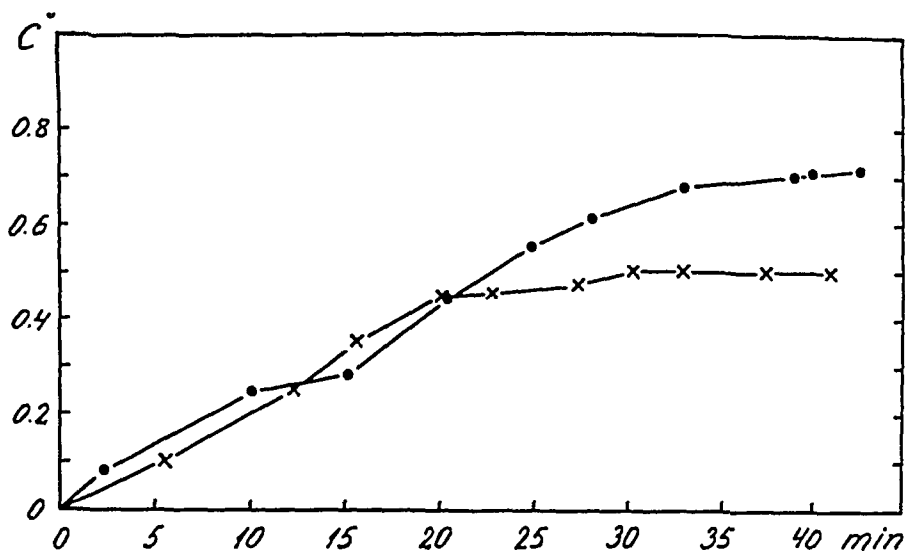


Fig. 1. Subj. P. Th. Increase in rectal temperature during 40 min of light work: ● —● with the legs at 7.2 Cal/min and × —× with the arms at 7.2 Cal/min. Heat production 5.9 and 6.4 Cal/min respectively.

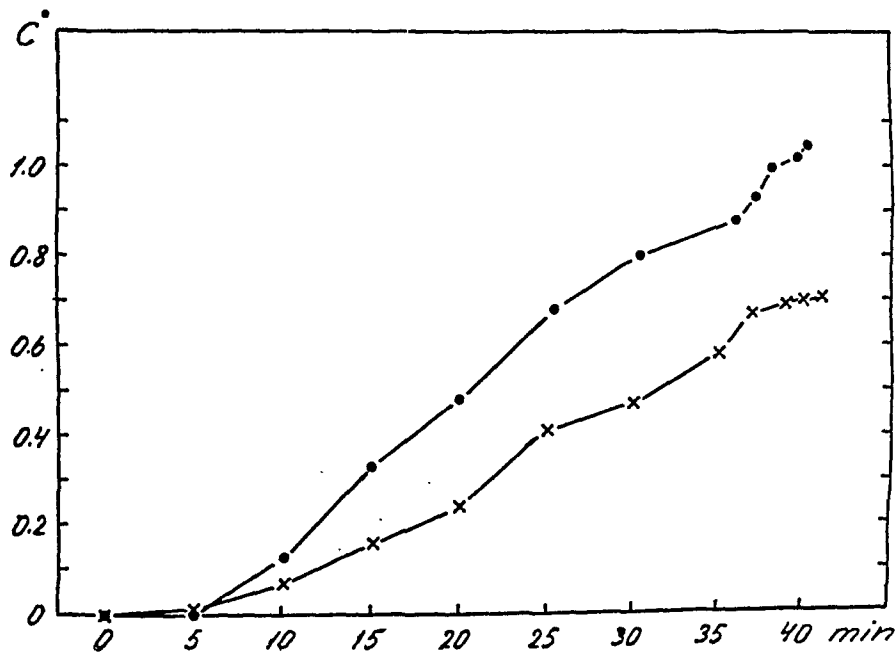


Fig. 2. Subj. O. W. Increase in rectal temperature during 40 min of heavy work: ● —● with the legs at 14.35 Cal/min and × —× with the arms at 14.30 Cal/min. Heat production 11.50 and 12.22 Cal/min respectively.

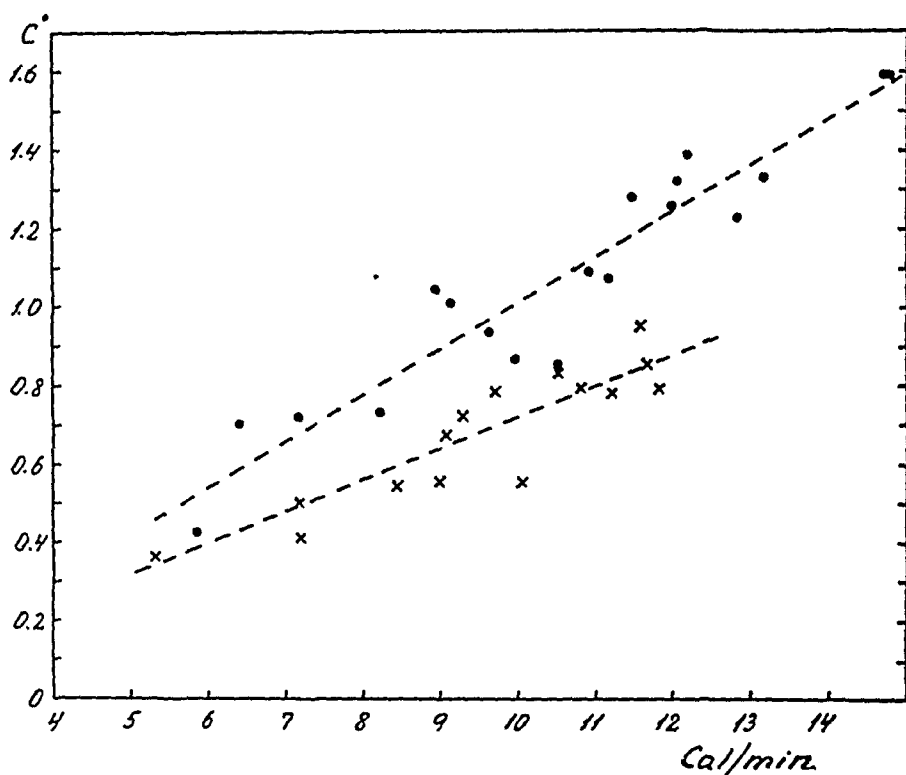


Fig. 3. Subj. P. Th. Increase in rectal temperature after 40 min of work in relation to total energy output in Cal/min.

● — ● leg-work.
 × — × arm-work.

start and not swallowed again until about 10 minutes before finish. The results from 10 experiments of both kinds on O. W. are shown in table 2. It can be seen that the nearness of the measuring thermocouple to the working muscles during leg-work has had no effect on the increase in temperature, which is higher than during work with the arms also when measured in the ventricle.

A few experiments, 4 of each kind on P.Th. showed the same viz. that the increase in temperature measured in the stomach was higher during leg-work than during arm-work.

The results of these experiments, therefore, seem to show conclusively that *work at a given energy output, or at a given heat production, creates a larger increase in body temperature in leg-work than in arm-work.*

Work costing the same energy output is felt subjectively much harder when performed with the arms than when performed with

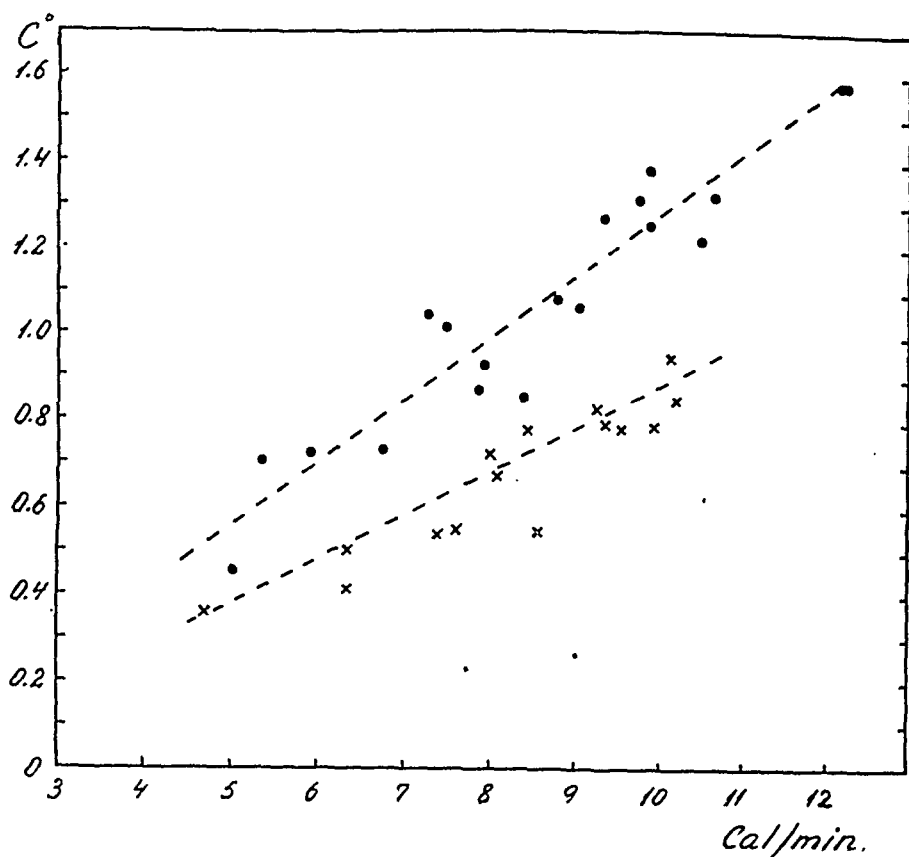


Fig. 4. Subj. P. Th. Increase in rectal temperature after 40 min of work in relation to heat-production in Cal/min.

● — leg-work.
 × — arm-work.

the legs. The ventilation, the pulse rate and the level of the blood lactates will also be considerably higher during arm-work.

As some of these factors might influence the level of the body temperature, some experiments were made, in which work with the legs was performed at a lowered barometric pressure, viz. corresponding to an altitude of 4,000 m, in a low-pressure chamber. Work at this altitude at the rate of 1,100 mkg/min was felt subjectively as very hard, and ventilation, pulse rate and blood lactates rise to levels considerably higher than at normal pressure. The effect on the increase in body temperature, as compared to control experiments made in the same chamber but at normal barometric pressure, is shown in table 3.

It will be seen that the increase in temperature at 4,000 m is the same as at sea level.

Table 1.

Subj. O. W.	Total energy output Cal/min	Heat production Cal/min	Temp. at start C°	Temp. after 40 min C°	Increase in temp. C°
Work with legs (mean of 20) range ¹	12.2 8.8—15.7	10.2 7.1—12.5	37.09	37.84	0.75 0.36—1.37
Work with arms (mean of 19) range ¹	11.8 8.3—14.9	10.1 7.1—12.8	37.08	37.55	0.47 0.18—0.90

Table 2.

Subj. O. W.	Total energy output Cal/min	Heat production Cal/min	Rectal temp. at start C°	Ventricle temp. at start C°	Increase in rect. temp.	Increase in vent. temp.
Work with legs (mean of 10) range	13.5 11.9—15.7	10.8 9.6—12.5	37.07	36.97	0.93 0.70—1.37	0.93 0.65—1.26
Work with arms (mean of 10) range	13.1 11.6—14.9	11.2 9.9—12.8	37.05	37.02	0.63 0.35—0.90	0.73 0.47—1.00

Table 3.

Subj. O. W. 1,100 mkg/min	Total energy output Cal/min	Heat production Cal/min	Rectal temp. at start C°	Increase in temp. C°
Sea level (mean of 6) range	13.1 12.6—13.5	10.5 10.0—10.9	37.08	1.02 0.84—1.29
4,000 m (mean of 6) range	13.3 12.6—13.9	10.7 10.3—11.3	37.11	1.09 0.96—1.26

Discussion.

The results of the experiments presented above corroborate the conclusion of NIELSEN, viz., that the higher temperature during

¹ The wide range is due to the effect of training which increased the subject's capacity for arm-work considerably.

work is not due to an inability of the heat-dissipating mechanism in counteracting the increased formation of heat. For if this were so, why, then, should calories produced in the leg muscles be more difficult to get rid of than calories produced in the muscles of the upper limb? The heat-dissipating centre must be "set" at another level during work than in rest, and the "setting" must be induced by some factor related to the work, and — according to the present experiments — different for arm-work and for leg-work.

Factors, related to the work, and different for arm-work and leg-work may be partly of nervous origin, partly of chemical nature. Among the first may be mentioned the subjective feeling of exertion or strain. This feeling is much stronger during arm-work than during work with the legs and might — for instance by causing an overproduction of sweat — explain the lower temperature during arm-work. But the experiments at 4,000 m (table 3), where this feeling was considerably stronger than at sea level, showed no effect on the level of the body temperature.

Whether the cortical motor impulses, directly or by irradiation, influence the heat-dissipating centre cannot be decided, but the possibility exists, and it is also very well possible that the integrated proprioceptive reflex impulses from legs and from arms vary and therefore may influence the heat-centre differently. A parallel to this latter may be found in the difference between the ventilation in arm-work and in leg-work at low intensities of work, as suggested by ASMUSSEN and NIELSEN (1946).

Among the factors of *chemical* nature that differ in arm-work and in leg-work are in first line those related to the different degree of anaerobiosis in the two kinds of work. These factors have been shown to play an important rôle in the regulation of ventilation during heavy work (ASMUSSEN and NIELSEN) and might easily be assumed, also, to influence the heat-centre. But then again the experiments at high altitude contradict the simple assumption that metabolites from the anaerobic processes should govern the "setting" of the heat-centre. For, as shown by ASMUSSEN and NIELSEN and recently by LUNDIN and STRÖM (1947), the level of the blood lactates, which can be taken as an expression of the anaerobicity of the work, is higher the lower the O_2 -tension and, as seen from table 3, the increase in temperature is the same at 4,000 m at a low pO_2 as at sea level.

Another factor that may influence the body temperature differently in the two kinds of work is the distribution of the blood.

While very little if any extra blood accumulates in the legs while bicycling [ASMUSSEN (1943)] a certain amount of blood will probably pool in the veins of the legs while sitting on the high stool during arm-work. Such a changed distribution of the blood in the periphery has been shown by NIELSEN, HERRINGTON and WINSLOW (1940) to influence the rectal temperature, but in opposite direction of what is found in the present experiments.

An effect of the position on the general circulation could not be detected: the cardiac output, determined during arm-work and leg-work on P.Th. with the acetylene-method showed no difference, although the pulse rate was somewhat higher during work with the arms, possibly because of an accumulating of blood in the legs under the conditions of the experiments as mentioned above. An eventual effect of the different rates of ventilation in the two kinds of work can also be ruled out, as show the experiments in high altitude, where the ventilation is greatly augmented. Errors due to the placing of the thermo-couple in the rectum, near the working muscles of the legs, can as mentioned be excluded from consideration, as the temperatures measured in the stomach showed the same increase as in the rectum (table 2).

Summing up, it might be said that besides differences in the nervous impulses to or from the muscles of the legs or the arms, none of the factors known to differ in exercise performed with legs or arms can be assumed to be responsible for the difference in the regulation of the temperature in the two kinds of work. It may, therefore, tentatively be assumed that the difference in the "setting" of the heat-dissipating centre in the two kinds of work is brought about either by irradiation of the efferent impulses from the motor-cortex, or by a summation of proprioceptive impulses arising in the working muscles.

The regulation of the body temperature during work may then be outlined as follows: The heat-dissipating centre is stimulated by the increased temperature of the blood, warmed in the working muscles. The centre sends out impulses by which the secretion of sweat and the blood flow through the skin is increased, until a balance between blood temperature and heat dissipation is reached. The temperature at which this balance is reached is, however, dependent on the sensitivity of the centre, and this in turn may among other factors be determined by the degree of the nervous activity including nervous impulses, reaching the centre from the periphery and, possibly, from the cortical motor areas. The peri-

pheral impulses might include sensations from the skin as well as proprioceptive impulses from muscles and joints. The integration in the centre of these impulses might contribute to the final "setting" of the body's "thermostat".

Summary.

Experiments were performed in which the increase in rectal temperature was measured during ergometer exercise with the arms and with the legs.

It was found that at a given output of energy, and at a given production of heat, the increase in temperature was larger with leg-work than with arm-work.

Temperature measurements in the stomach showed that this difference was not due to the proximity during leg-work of the working muscles to the measuring thermocouple.

Experiments with leg-work at sea level and at a simulated altitude of 4,000 m showed no difference in the temperature increase, indicating that a more anaerobic condition and a more pronounced subjective feeling of strain have no effect on the thermoregulating centre during work.

It is concluded that the different "setting" of the heat dissipating centre during work with the arms and with the legs possibly is due to a different set of nervous impulses, efferent and reflex, reaching the centre during the two kinds of work.

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Some Investigations of Erythropoiesis in Human Bone-Marrow Cultivated in Various Media.

By

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In previous reports (PLUM, 1946, 1947, 1942, 1943) methods are described for the determination of both the rate of erythrocyte formation in a bone-marrow suspension, and the influence of various substances on the rate of ripening of reticulocytes. The technique used in the former investigations is a modification of that of Osgood (1936), and is described in detail elsewhere (PLUM 1946, 1947). The growth and multiplication of the bone-marrow cells takes place under conditions as nearly normal as possible as regards pH, supply of nutrient substances and oxygen, and removal of waste products.

In these experiments (1947) a principle in the serum (or plasma) was found to be necessary for the normal development of the red blood corpuscles. It was therefore planned to do various experiments to elucidate this phenomenon: viz. to study the effects of different preparations used in the treatment of pernicious anemia, and to correlate the behavior of both normal and pathological bone marrow cells toward "foreign" and "native" sera. (The term "native" serum is used here to denote serum obtained from the subject who contributed the bone marrow. The "foreign" serum was obtained from another individual, who may or may not have been a normal subject, as noted in the text.) These studies form the basis of the present report.

Methods.

The bone-marrow was obtained by sternal puncture. Smears were made, and the remainder of the bone-marrow in the syringe was mixed with 0.5 ml of 3.0 % Na-Citrate. As soon as possible — within one-half hour — this sample was centrifuged and nucleated cells separated from most of the erythrocytes, ready for cultivation. (PLUM, 1946.) Before doing the sternal puncture, a 50 ml sample of blood was taken from the cubital vein to get serum for the nutrition fluid.

From the number of new-formed cells and the number of normoblasts present, one can calculate the average number of red cells formed from each normoblast per hour. Using this and other procedures, the author has shown that the erythrocytes are formed by budding of protoplasm from the normoblast, and not by mitosis and denucleation of the cells. Further details regarding the calculation are given in *Blood: the Journal of Hematology*, 1947.

In the experiments to be described, the following solutions were used as nutrition fluids.

- 1) Locke's fluid.
- 2) Folic acid, Lederle — 0.1 %.
- 3) Liver-extract, Hepsol fortior "MCO", 1.0 % (diluted with Locke's fluid).
- 4) Liver-extract, Hepsol fortior "MCO", 1.0 %, Tyrosine 0.01 % (diluted with Locke's fluid).
- 5) The subjects own serum, 1.0 %, diluted with Locke's fluid. (= "Native" serum).
- 6) Serum from another person, 1.0 %, diluted with Locke's fluid. (= "Foreign" serum).

Results of the Investigations.

I. Normal Material.

These investigations were carried out on bone-marrow obtained from medical students, nurses and industrial workers. All appeared to be in good health.

A. The Relative Effects of "Foreign" and "Native" Sera on Erythrocyte Formation.

The reticulocytes, or immature erythrocytes formed in the bone-marrow from normoblasts, will ripen both in marrow and peripheral circulation to mature erythrocytes. Previous studies have shown (PLUM 1942, 1943) that a principle is present in serum and in plasma which is necessary for the ripening of the reticulocytes. Studies were therefore made to determine whether the

principle responsible for this ripening could also accelerate the initial formation of the red blood corpuscles from the normoblasts.

About 25 experiments were carried out, employing blood and marrow from both male and female subjects, 24—44 years of age. Because of the increase in reticulocyte-ripening substances found in plasma during menstruation, female subjects were used, only 7—10 days after the end of their menstrual period.

During an experiment an increase takes place in the number of erythrocytes. This formation of erythrocytes takes place during the entire experimental period of 6 hours, and can be maintained longer. When Locke's fluid alone is used as the nutrient fluid (see below) the formation seems to stop after about 8 to 10 hours. For some hours thereafter there will be no change, or even a decrease, in the number of erythrocytes. After this interval, a secondary increase in the number of red cells may again appear. When liver-extract is added to the nutrient fluid, formation of erythrocytes proceeds for much longer than 8 hours before coming to a halt. It seems therefore, that there must be one or more principles in the normoblast which are able to activate the formation of erythrocytes for a while when certain essential substances (amino-acids etc.), are absent from the nutrition fluid. When this material is exhausted from the normoblasts, the formation of red cells stops, unless new material is added to the nutrition fluid.

As shown in Table 1, Locke's solution alone maintains only a slight production of red cells over a limited period of time. Both "foreign" and "native" sera in 1 % concentration, on the other hand, give rise to a rapid red cell production. Even here, however, there is a distinct and consistent difference, the "foreign" serum being about 15 % less active than the subject's own serum.

As indicated in column B, table 1, the average number of erythrocytes produced after addition of "native" serum is about 5.29 cells per nucleated cell per hour, which means that each normoblast produces an average of one erythrocyte every 10—15 minutes. At this rate it is possible to maintain the normal red blood cell picture in the human organism (C. M. PLUM, 1947).

The last 3 columns in this table show further the relative activities of Locke's fluid, "native" serum, and "foreign" serum. Thus, the first of these columns gives the per cent activity of foreign serum as compared to the "native" serum. The second and third columns give the relative activities of "native" and "foreign" sera as compared in each case to the activity of Locke's solution.

Table 1.

Number of Erythrocytes Produced per Normoblast per Hour in Normal Bone-marrow by using Locke's Fluid, "Native" Serum, and "Foreign" Serum as Nutrient Fluids.

Subject		Activity of Nutrition Fluid ¹			Relative Activities		
Sex	Age (years)	Locke's fluid (A)	"Native" serum (B)	"Foreign" serum (C)	$\frac{C \times 100}{B}$	$\frac{B - A}{A}$	$\frac{C - A}{A}$
Female	25	1.29	5.58	4.68	84	4.33	2.63
	26	1.04	5.02	4.47	89	3.82	3.30
	26	1.52	5.62	4.61	83	2.70	2.03
	27	1.42	4.99	3.47	87	2.52	1.45
	29	1.17	5.23	4.70	90	3.42	3.02
	31	1.02	5.17	4.18	81	4.07	3.10
	32	1.37	5.29	4.49	85	2.86	2.28
	32	1.23	4.85	3.97	82	2.94	2.33
	33	1.37	5.11	4.60	90	2.73	2.36
	35	1.13	4.64	4.13	89	3.11	2.66
	35	1.08	4.67	3.88	83	3.32	2.59
	38	1.33	5.30	4.60	87	2.98	2.46
	40	1.25	5.27	4.32	82	3.22	2.46
	40	1.38	5.56	4.94	88	3.03	2.60
Male	24	1.48	5.28	4.38	83	2.57	1.96
	25	1.20	5.43	4.89	90	3.53	3.08
	27	1.08	4.99	4.14	82	3.62	2.80
	27	1.23	5.37	4.68	87	3.37	2.80
	29	1.33	5.52	4.64	84	3.15	2.48
	30	1.04	5.12	4.45	87	3.92	3.28
	32	1.37	5.31	4.35	82	2.88	2.17
	32	1.40	5.48	4.60	84	2.91	2.58
	36	1.07	5.15	4.38	85	3.82	3.09
	40	1.09	5.02	4.52	90	3.61	3.06
	44	1.27	5.56	4.88	88	3.38	2.84
Maximum		1.52	5.62	4.94	90	4.07	3.30
Minimum		1.02	4.62	3.47	81	2.52	1.45
Average		1.26	5.29	4.50	85	3.25	2.66

The quantitatively different response found in the production of red cells when bone-marrow is activated by adding "foreign", as contrasted with "native" serum has been interpreted to mean that serum contains a principle which can be used only by the bone-marrow cells of the same person. An alternative possibility is that inhibiting materials are present in "foreign" sera.

¹ Results are expressed in terms of the average number of erythrocytes formed per normoblast per hour.

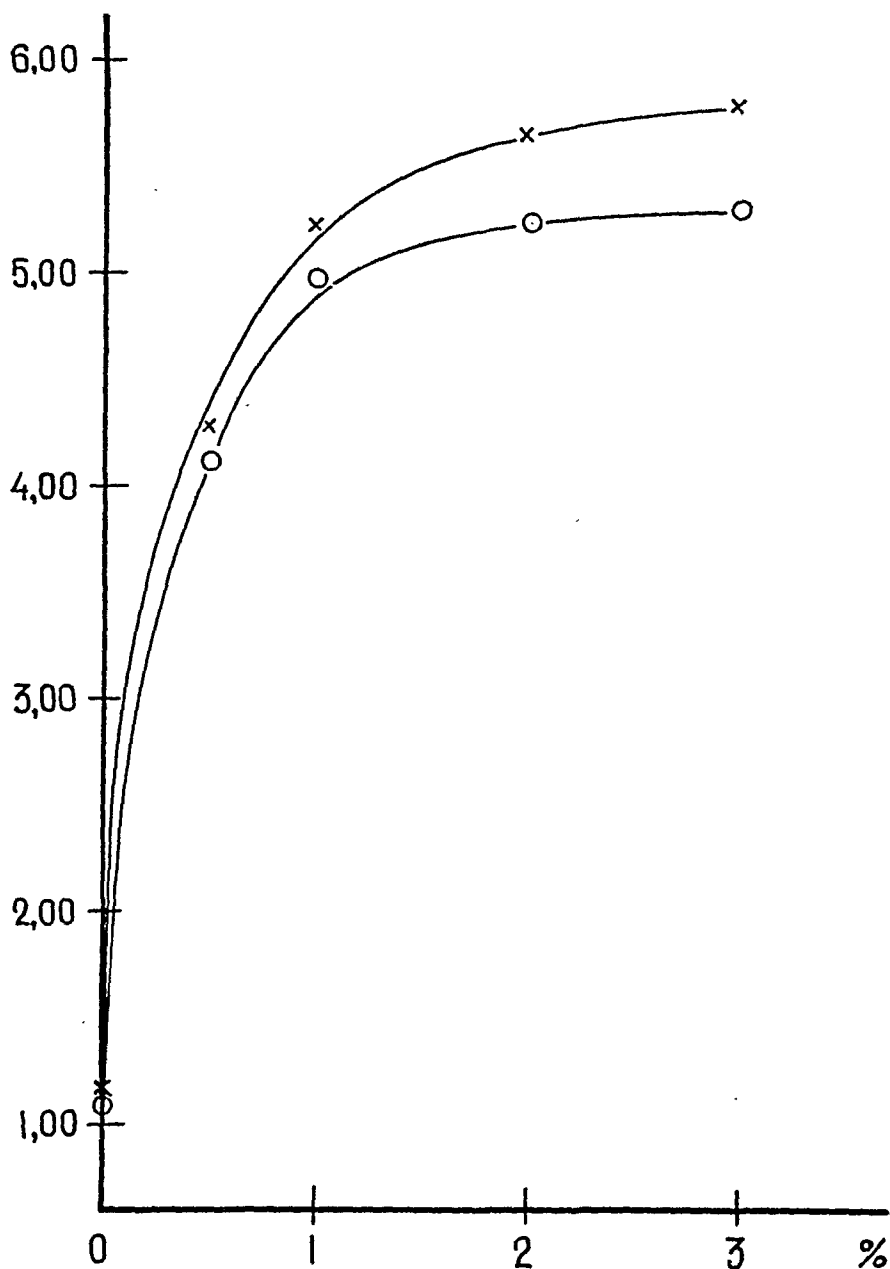


Fig. 1. The Relation between serum concentration and red cell production in a bone-marrow culture.

Ordinate: number of erythrocytes produced per normoblast per hour; Nutrient fluid: Locke's fluid. + Serum.

Abscisse: concentration of serum in the nutrient fluid.

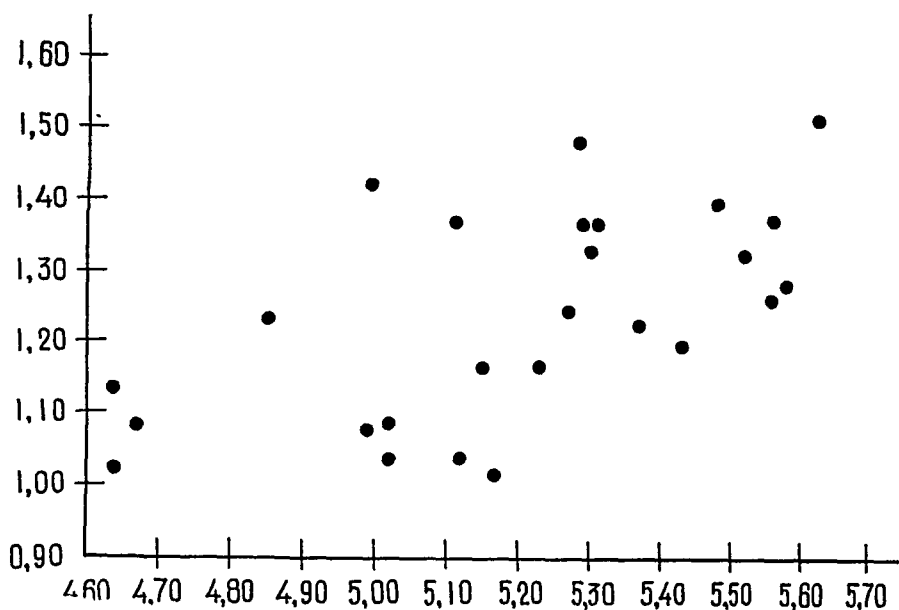


Fig. 2. The Relation between the Formation of Red Cells in a Bone-marrow Culture when the Nutrient Fluid is Locke's Fluid (Ordinate) and 1 % "Native" Serum (Abcisse). The ciphers in both ordinate and abscisse give the number of red cells produced per normoblast per hour.

A one percent solution of serum in Locke's fluid was used in these experiments since studies carried out with different concentrations of serum showed that the formation of red cells increases very little by using higher concentrations. These studies are summarized in Fig. 1.

From the data given in table I it is seen that there are fairly large individual differences in the rate of "spontaneous" formation of red cells when the nutrient fluid is Locke's solution. In Fig. 2 is plotted the relationship between this "spontaneous" formation of the red cells and the formation of the cells when a 1 % solution of "native" serum is added to the Locke's solution. It will be seen, in general, that a low rate of "spontaneous" formation of the red cells is associated with a slower rate of formation with added serum. The same relations have been demonstrated when the activation is produced by liver-extract (C. M. PLUM, 1947).

The results dealing with the "spontaneous" formation of red cells suggest that a principle in the normoblast may activate the production of red cells for some time. After the temporary cessation of red cell formation, the second increase in erythropoiesis seems to be associated with the destruction of some erythrocytes

and the liberation of some necessary principles from these cells. It should be pointed out, however, that the formation of the red cells in Locke's solution is not a natural one, since the cells formed do not grow to the normal size, such as is seen when serum is used as the nutrient fluid.

Because the normoblast itself in Locke's solution produces some erythrocytes (1.03—1.56, average 1.23, per normoblast per hour), it is always necessary to observe the rate of spontaneous formation of red cells, and to include this figure in the various calculations.

B. The Effect of Various Preparations on Erythrocyte Formation.

As noted above, results previously reported in this journal (C. M. PLUM, 1943) show that the ripening of reticulocytes into mature erythrocytes depends on some substances found in serum and plasma. Liver-extract too contains substances able to activate the ripening of the reticulocytes, and the effect on the ripening process is further increased by adding tyrosine to the liver-extract (C. M. PLUM, 1944). The effect of normal plasma or serum, on the other hand, is not enhanced by tyrosine. Because of these findings, it was desirable to investigate the effect of various materials on erythropoieses in bone-marrow culture, and specifically on the rate of formation of erythrocytes from normoblasts.

The effect of various preparations employed in the treatment of pernicious anemia was studied. These included folic acid, liver extract (Hepsol fortior, MCO), and liver extract plus tyrosine, in addition to "native" and "foreign" sera. These materials were each used in 1 % solution except for tyrosine which was used in a final concentration of 0.01 %. Controls employing only Locke's fluid were used in all instances.

Results of the 25 experiments done are summarized in table 2. The data for each of the added materials are given first in terms of the average red cell production per normoblast per hour (B) and then, (underlined) in terms of the ratio of its activity alone (B—A) to that of Locke's solution alone (A). This latter ratio (B—A/A) is similar to that used in the final 2 columns of table 1.

From this table it is apparent that each of the added materials increases the rate of red cell production appreciably over that found with only Locke's solution, though to a different degree. Greatest activity was produced by native serum where the average

ratio of B—A/A was 3.25. Folic acid, with an average ratio of 2.13 was least active. "Foreign" serum (2.66) and liver extract, with (2.58) and without (2.66) the addition of tyrosine had intermediate and about equal activities.

Table 2.

Investigations of Normal Bone-marrow Supported by Various Preparations Used in the Treatment of Pernicious Anemia.

The italics refer to the index described in the text, the other ciphers refer to the number of red cells produced per normoblast per hour. The results for the different media used as nutrient fluid seem to be correlated in such a way that a maximum in the spontaneous formation (Locke's fluid) follows a maximum in the activated formation of cells (Folic acid, Liver-extract etc.).

The results here are based on 25 different examinations.

Nutrient fluid	Locke's fluid	Folic acid 0.1 %		Hepsol fort. 1 %		Hepsol fort. 1 % + tyrosine, 0.01 %		"Foreign" serum 1 %		"Native" serum 1 %	
Maximum	1.56	5.80	2.72	6.51	3.17	6.58	3.22	4.94	3.30	5.62	4.07
Minimum	1.03	2.71	1.62	3.16	2.04	3.07	1.98	3.47	1.45	4.62	2.52
Average	1.23	3.85	2.13	4.50	2.66	4.41	2.58	4.50	2.66	5.29	3.25

Two of these results are of especial interest in comparing erythrocyte formation with reticulocyte ripening. First, liver-extract and folic acid are more effective than serum in the latter case, whereas the opposite is here shown to be true of the former process. Secondly, tyrosine increases the reticulocyte ripening effect of liver-extract, but has no effect on the rate of formation of red cells under the conditions employed.

The conclusions to be drawn from these experiments then are these: (1) that folic acid is a powerful stimulant of red cell formation; (2) that liver-extract made from oxen, and serum from other individuals supply one or more further active substances but they may lack a factor which can be supplied only by "native" serum, or by red cell products as indicated by the secondary formation of erythrocytes in late stages of experiments with Locke's solution, and (3), that there are some differences between those factors responsible for red cell formation and those responsible for reticulocyte ripening.

II. Pathological Material.¹

The investigations were carried out on patients with different diseases, but chief emphasis was given to patients with blood diseases.

The problems were three-fold: (1) Does the pathological marrow react in a normal way to substances able to activate a normal marrow, 2) does the pathological marrow react in a normal way to normal serum, and 3) does normal marrow react in a normal way to pathological serum?

44 pathological cases were examined in an attempt to answer the first of these questions. The results are given in table 3. The spontaneous formation of red cells in the bone marrow suspension was reduced only in several cases of leukemia myeloides, in the 1 case of leukemia lymphaticus, and in the cases of untreated pernicious anemia. In all other pathological cases investigated it is impossible to state anything definite regarding results with Locke's solution, either because only a single case has been investigated or because the results are too divergent.

A. The Effect of Folic Acid, Liver-Extract etc.

It was found that in most of the cases investigated the formation of red cells can be activated at least to the same degree as in cultures of normal marrow. Only in lymphogranulomatosis benigna is the activating effect of folic acid or of liver preparations negligibly small. In most cases studied and especially in pernicious anemia both before and after therapy, the activating effect of folic acid was actually considerably greater than that seen in the average normal bone marrow (P. A. average = 2.74; Normal average 2.13). This difference cannot be an artifact due only to the low value of "A", since "A" in untreated P.A., averaged 0.76, while it averaged 1.20 in treated P.A., yet the average ratio B—A/A in the former was 2.70 and in the latter it was 2.76.

Some of the cases investigated were examined several times during the period of treatment. Results indicate that in myeloid leukemia there is an increase in the "spontaneous" formation of red cells following the addition of liver extract to the bone marrow

¹ I am indebted especially to Professor C. SONNE, M. D., and Professor E. WARBURG, M. D., The University Clinics A and B, Rigshospitalet, Copenhagen, for supplying the bone-marrow samples for the investigation.

Table 3.

Studies of Erythropoiesis in 44 Pathological Bone-marrow.

Date 1946	Jour.	Age	Sex	Diagnosis	Locke's Fluid	Folic acid 0.1 %	Hepsol fort. 1 %	Hepsol fort. 1 % + tyrosine 0.01 %
26.4	520B	35	F	Lymphogranulomatosis benigna	1.18	1.89	1.63	1.81
29.4	B		M	"	0.81	1.34	1.16	1.25
12.9	80B	17	M	"	0.93	1.83	1.47	1.53
31.5	568B	48	"	"	1.12	1.63	1.85	1.94
6.5	977B	37	"	"	1.14	1.79	1.72	1.90
20.6	869A	46	"	"	1.12	1.95	1.79	1.88
23.5	626B	59	"	Morb. Basedowii	1.20	2.16	2.34	2.64
14.5	612A	27	F	" ant.	0.82	1.82	1.94	2.33
16.7	861A	67	"	Myxedema, anemia simplex	1.50	2.87	2.88	2.79
5.9	832B	73	M	Myelomatosis	0.99	2.38	2.48	2.40
2.5	395B	58	"	"	1.16	2.32	2.46	2.32
11.6	Bldg.	71	"	"	1.05	2.48	2.63	2.66
22.7	811B	72	"	Leukosis myeloides	0.84	3.02	1.75	2.73
19.8	"	"	"	" (after treatment)	1.25	2.76	2.60	2.67
2.9	"	"	"	"	1.00	2.98	2.21	2.35
18.8	862B	69	F	"	0.98	2.79	2.08	2.13
2.8	892B	58	"	"	0.93	2.86	1.93	2.52
22.8	"	"	"	"	0.99	2.68	1.79	2.29
31.5	735A	66	"	"	0.78	2.63	1.90	2.31
26.6	"	"	"	"	1.05	2.71	2.38	2.49
12.7	"	"	"	"	1.38	2.85	2.73	2.80
17.10	"	"	"	"	1.27	2.46	2.56	2.43
22.7	769B	37	M	Leukosis lymphatica	0.87	2.69	2.00	2.21
17.6	913A	43	F	"	1.13	2.20	2.43	2.41
21.6	"	"	"	" (after treatment)	0.82	2.13	2.11	2.23
29.8	"	"	"	"	0.95	1.93	2.00	2.26
14.10	"	"	"	"	1.08	2.34	2.37	2.57
27.8	Rst. 411	92	"	"	0.88	1.67	1.09	1.90
29.4	566B	53	M	Leukosis aleukemia	0.88	1.95	1.99	1.91
15.5	"	"	"	"	0.92	2.12	2.04	1.87
4.6	"	"	"	"	0.90	2.00	2.12	2.12
10.5	166A	66	"	Anemia hyperchr. Leukemia aleukemia	0.73	2.47	1.47	1.98
20.5	"	"	"	"	0.69	2.56	1.53	1.98
5.7	707A	73	F	Anemia hyperchr.	0.98	2.69	2.00	2.23
2.7	678A	40	"	"	0.91	2.79	2.08	2.27
15.5	E. A. St.	77	"	Pernicious A. (treated)	1.24	2.79	1.69	2.09
15.5	A. P.	83	"	" (treated with liver)	1.13	2.87	1.62	2.45
31.5	"	"	"	" (treated with liver + Tyrosine)	1.20	2.78	2.50	2.65
6.5	V. J.	74	"	Pernicious A. (treated with liver)	1.07	3.06	2.78	2.86
9.7	N. J.	63	"	"	1.12	2.56	2.60	2.78
11.6	J. C.	53	"	Pernicious A. (untreated)	0.69	2.56	1.99	2.40

Table 3 (cont.).¹

Date 1946	Jour.	Age	Sex	Diagnosis	Locke's Fluid	Folic acid 0.1 %	Hepsol fort. 1 %	Hepsol fort. 1 % + tyrosine 0.01 %
25.6	J. C.	53	F	Pernicious A. (treated with liver)	1.29	2.46	2.33	2.39
7.8	A. L.	69	M	Pernicious A. (untreated)	0.79	2.83	2.24	2.56
21.8	"	"	"	Pernicious A. (treated with folic acid)	1.42	3.00	2.62	2.83
9.9	M. F.	64	"	Pernicious A. (untreated)	0.81	2.71	2.39	2.66
1.10	"	"	"	Pernicious A. (treated with liver)	1.12	2.48	2.49	2.51
9.5	694A	21	F	Anemia (typus incertus). Morb. coeliacus ant.	0.67	3.26	1.03	2.77
14.5	531B	45	M	Ulcus duodeni c. anemia	1.30	2.14	2.44	3.00
18.7	712B	63	F	Anemia simplex, febris undulans	0.96	3.09	2.85	2.89
11.7	946B	56	"	Anemia simplex, nephritis chr.	1.18	2.41	2.64	2.54
4.6	663A	17	"	Osteomyelitis clav. sin. anemia simpl.	1.20	2.31	2.53	2.68
16.5	610B	56	M	Morb. cord. mitral.	1.36	2.31	2.56	2.65
26.5	802B	53	"	Carc. bronch. pulm. et met.	1.00	1.76	1.48	1.84
15.8	797B	63	"	Carc. pulm.	1.11	2.72	2.56	2.65
9.9	601B	66	"	Carc. prostatae, anemia sec.	1.00	1.83	1.76	2.00
20.5	525B	47	F	Carc. pancreatic.	1.15	2.42	2.53	2.69
4.9	C	43	M	"	0.91	1.26	1.12	1.24
4.7	778B	71	F	Hepatitis subacuta	1.23	2.16	2.41	2.18
27.8	977A	68	M	Sepsis lenta (strept. hemol.)	1.27	2.05	2.43	2.21
				Maximum	1.56	2.72	3.17	3.22
				Average	1.23	2.13	2.66	2.58
				Minimum	1.03	1.62	2.04	1.98

culture when the number of leucocytes is reduced by treatment.² Interestingly, too, the untreated cases show a suboptimal effect with liver which is increased by tyrosine. After treatment of the 2 patients thus studied, liver extract alone gave an optimal effect. The same

¹ Results are given in terms of the ratio B—A/A, described above.

² The cases 811B and 769B are published in detail by V. MORTENSEN, M. D., Ugeskrift f. Læger, 1947, but without the results of the bone marrow activity measured in the culture. The cases 735A and 913A are to be published by J. CLEMMESSEN, M. D., T. ESPERSEN, M. D., and C. M. PLUM, Ph. D., in Blood: Journal of Hematology.

picture appears to be true of the one case of leukosis lymphatica studied.

In the cases with simple anemia or anemias resulting from cancer, it appears that there are no certain differences from the normal.

From the above results it can be seen therefore that the normoblasts in most of the bone-marrow investigated are able to respond normally to folic acid, liver-extract, or liver-extract plus tyrosine.

B. Cross-Correlation Experiments.

Cross-correlation experiments were done to get an answer to questions 2 and 3, namely, to determine whether the disease was due primarily to a deficiency of the marrow cells or of the serum. In these experiments marrow from one person (A) was stimulated by serum from both A and from another person B. Similarly, the marrow from B was stimulated by serum from both B and A. Typical results of such cross-correlation experiments on normal persons are given in table 4. These results agree with those of table 1 in indicating a greater activating effect by "native" than by "foreign" serum.

Table 4.

Cross-correlation Experiments with Normal Marrow and Normal Serum.

	Normal serum (A)	Normal serum (B)		Normal serum (C)	Normal serum (D)
Normal Marrow (A)	5.37	4.41	Normal Marrow (C)	4.98	4.48
Normal Marrow (B)	4.42	5.02	Normal Marrow (D)	4.53	5.52

In table 5 two examples are given of cross-correlation experiments carried out between normal and pathological marrows. In the first example cited, the abnormality in pernicious anemia is shown to reside in a plasma deficiency rather than in a cellular deficiency. In the case of myeloid leukemia both serum and cells are shown to be deficient.

By using the schema given in table 4 and 5 one can draw up certain quantitative relationships between the normal and patho-

Table 5.

Cross-correlation Experiments with both Normal and Pathological Sera and Bone-marrow.

	Normal serum (E)	Pernicious Anemia serum		Normal serum (F)	Leucosis myeloid serum
Normal Marrow (E)	5.39	3.45	Normal Marrow (F)	5.16	3.20
Pernicious Anemia marrow	5.26	3.88	Leucosis myeloid marrow	3.97	3.35

logical marrows and sera. These can be expressed in the following 3 ratios:

1) $\frac{\text{Pathological marrow added to "native" serum}}{\text{Normal marrow added to "native" serum}} = \text{the total relative erythropoietic activity of the pathological marrow and serum.}$

If the red cell formation is normal, this ratio should not differ significantly from unity (0.95—1.06).

2) $\frac{\text{Pathological marrow added to normal serum}}{\text{Normal marrow added to "native" serum}} = \text{the relative activity of the pathological bone marrow cells: If nothing is wrong with the marrow in the pathological case, the ratio should not differ significantly from 0.85 (0.80—0.90).}$

3) $\frac{\text{Normal marrow added to pathological serum}}{\text{Normal marrow added to "native" serum}} = \text{the relative activity of the pathological serum: A significant reduction of the ratio below 0.85 indicates a diminished function of the serum from the pathological case.}$

The results given in table 6 are based upon these 3 ratios. In the one case of leukemia studied here it will be seen that there was a slight increase in the total cell plus serum function following the treatment. Analogous results are found in table 3, where the bone-marrow function is activated by folic acid etc. It will be seen too, that prior to treatment both serum and bone-marrow cell activity are decreased (22/7). After the treatment with urethane a decrease took place in the peripheral white cell count and following this the activity of serum increased (19/8). When the number of white

Table 6.

Further Results of Cross-correlation Experiments.

The figures in column I represent the activity of the total pathological Bone-marrow: serum system corresponding to relation 1 (see text); column II = the activity of the pathological bone-marrow cells (relation 2); column III = the activity of the pathological serum (relation 3). All the values are corrected to the average of the normal red cell production: 5.29 (see table 1).

Date	Journ.	Age	Sex	Diagnoses	I	II	III
22. 7	811B	72	M	Leukosis myeloides	0.65	0.79	0.64
19. 8	»	»	»	» » (after treatment)	0.72	0.80	0.77
2. 9	»	»	»	» »	0.72	0.75	0.74
25. 6	J. C.	53	F	Pernicious anemia (untreated)	0.71	0.92	0.64
7. 8	»	»	»	» » (treated with liver)	0.92	0.87	0.86
9. 9	M. F.	64	M	» » (untreated)	0.59	0.98	0.61
1. 10	»	»	»	» » (treated with liver)	0.88	0.90	0.85
7. 8	A. L.	69	»	» » (untreated)	0.58	0.98	0.64
25. 8	»	»	»	» » (treated with folic acid)	0.89	0.87	0.83

cells increased, however (2/9), cell and serum activities decreased slightly.

In all the cases of untreated pernicious anemia the serum function is reduced, whereas the function of the bone-marrow cells is slightly increased. After treatment with liver and folic acid and after the reticulocyte crisis there will be found an increase of the total function of the bone-marrow, the function of the bone-marrow cells and of the serum.

These preliminary investigations carried out on cases of leukemia and pernicious anemia seem to show that similar disorders of erythropoiesis may appear in these diseases, and that the erythropoietic system lacks some serum-principles in both conditions. These may or may not be the same. Further investigations are proceeding on this problem.

Summary.

Experiments have been performed on human sternal marrow kept alive in vitro in Locke's solution with and without the addition of different antipernicious anemia preparations or of serum from either the same or from another person. These materials have been shown to maintain erythropoietic activity at various levels.

The slight formation of red cells which takes place in Locke's solution comes to a standstill after 8—10 hours, but is resumed

later when red cells are hemolyzed and presumably liberate nutritive materials. Folic acid (0.1 %) will increase the formation of red cells considerably and liver extract even more so. "Foreign" serum has about the same effect, but the subject's own serum causes most rapid erythropoiesis, thus pointing to the existence of a factor peculiar to each individual. A number of pathological cases were investigated. The "spontaneous" production of the red cells was diminished in untreated pernicious anemia and leukemia, but was normal in various other conditions studied. In most of the cases, the pathological marrow responded quite as a normal marrow does when liver or folic acid is added to the nutrient fluid. Only in lymphogranulomatosis benigna was a significant decrease below normal found in the production of red cells when these materials were used. Tyrosine increased the effect of liver extract in untreated pernicious anemia and in leukemia but had no added effect on normal bone-marrow.

The production of red cells from the pathological marrow nourished by normal serum is slightly decreased below normal in some of the pathological cases, especially in leukemia. In this disease and in pernicious anemia there was a decreased total function of bone-marrow plus "native" serum. All pathological marrows, however, were considerably activated by the addition of normal serum to the nutrient fluid. The use of pathological serum with normal marrow results in a significant decrease in red cell production over that found when normal marrow is activated by "foreign" normal serum.

In patients in whom a decrease was observed in the activity of the bone marrow cells and of the serum, *i. e.* in pernicious anemia, effective therapy resulted in an increase in the amount of those substances in both cells and serum which activate the formation of red blood cells in the marrow cultures.

These results considered together with the results found in investigations of the ripening of the reticulocytes *in vitro* show that there are certain active principles in the serum, some being necessary for the production of red cells and some for the ripening of the reticulocytes. It appears that certain of these principles are necessary for both processes.

I extend my deep gratitude to the Carlsberg Foundation and to the King Christian the Tenth Fund for supporting these investigations.

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SELECTIVE RESPONSES
TO THERMAL STIMULATION OF
MAMMALIAN NERVES

By

CURT VON EULER

Stockholm 1947

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Preface

Observations in this laboratory had drawn attention to various possibilities inherent in the application of thermic stimuli to nerve fibres, particularly with regard to selective stimulation of small fibres. For this reason it was felt that thermostimulation of nerves, a very much neglected subject, was a theme that deserved a broad experimental survey. This work was undertaken by myself in the early autumn 1945. Its result forms the subject of this thesis.

It is a great pleasure for me to express my sincere gratitude to the Director of the Nobel Institute for Neurophysiology, Professor RAGNAR GRANIT, for valuable advice, criticism and encouragement during these years.

I also wish to convey my heartfelt thanks to Professor ULF VON EULER for a number of valuable suggestions and for his kindness in placing at my disposal instruments and facilities in his laboratory.

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Stockholm, March 1947.

CURT VON EULER.

Introduction and problem

This work deals with an analysis of selective responses obtained by thermal stimulation of mammalian nerves.

It arose as a consequence of previous experiments carried out in this laboratory with nerve as model sense-organ. In their paper on Nerve as Model Temperature End-Organ, BERNHARD and GRANIT (1946) found that when a cooled region of a nerve became electronegative relative to the rest of the nerve (GRÜTZNER, 1881; VERZÁR, 1911, see Historical section) an impulse discharge started soon after the rise of this potential. They also demonstrated that when the nerve was locally heated to about 45° the heated region similarly became electronegative so that nervous tissue thus was found to behave as a 'thermocouple' having its zero point at body temperature. The negativity in response to heating was likewise accompanied by a discharge of impulses. These temperature potentials were therefore regarded as the 'generator potentials' responsible for the impulse activity, in accordance with the views advanced by BERNHARD, GRANIT and SKOGLUND (1942). It was further observed that the animal responded differently to heating and cooling of the sciatic nerve: only upon heating were there signs of struggling as if in response to painful stimuli.

This observation suggested that cooling and heating excited different afferent fibres. In an old paper by GRÜTZNER (1878), containing a number of good observations, it had been claimed that local heating stimulated the whole fibre spectrum of afferents but practically no efferent fibres and that local cooling did not excite any fibres at all.

It is clear that every method of selective nerve fibre stimulation would be a valuable asset, especially one, by which only the thinnest fibres were excited. If selective enough such a method would facilitate investigation of problems associated with the thinnest fibres and their central connections and, consequently

be of importance for the neurophysiology of the nervous system as a whole.

Experimentation was begun in the autumn 1945 and has led to a number of results of which, in order to facilitate perusal of this paper, the following ones deserve to be specially emphasized.

It has been established that heat and cold are relatively selective stimuli for small and large fibres and that stimulation of afferents with heat sets up a typical nociceptive reflex. Fibre differentiation by temperature changes proved, however, to have further interesting and new aspects. It was thus found that there are characteristic differences between vegetative and somatic fibres of the same size with respect to thermosensitivity. Finally it proved possible to find fibres in which the thermopotentials to heat and cold were differentiated in such a manner that certain nerves responded to cooling, others to heating.

Historical Section

Thermal stimulation and temperature potentials.

From the middle of the last century onwards there are several papers on thermal stimulation of frog nerves. In most of them burning hot temperatures were used. LAUTENBACH (1879) discussed the inconsistent results obtained with moderate temperatures and stated that frogs of different breeds varied greatly in their sensitivity to thermal stimulation, similarly frogs taken in different seasons (see also NOVIS, 1945).

PICKFORD (1851) made the interesting observation (on frogs) that while excitability within certain limits varied with the temperature, excitation could only be evoked by sudden temperature changes of positive or negative direction. Before the work of BERNHARD and GRANIT (1946), mentioned above, there is only one systematic study of temperature stimulation on mammalian nerves, namely that of GRÜTZNER. GRÜTZNER and HEIDENHAIN (1878) had observed, during work on vasomotor reflexes, that nociceptive reflexes with marked increases in blood pressure were elicited by heating a peripheral nerve. GRÜTZNER (1878) investigated the thermal excitability of different nerves in the dog and the rabbit and found that vasomotor reflexes could be evoked from the central end of somatic and visceral nerves. By thermal stimulation of the peripheral end of severed somatic and visceral nerves it was impossible to obtain effects from muscles or viscera. However, when the heating of the peripheral end of the severed sciatic nerve was carried to 50° a vasodilatation of the vessels in the skin of the leg was noted. From these observations, some of which I have been able to confirm in my paper, GRÜTZNER reached the conclusion that "durch Erwärmung auf 45° — 50° C erregt werden die centripetalen Nerven der verschiedensten Art während mit Ausnahme der Hauterweiterer die centrifugalen in ihrer Erregbarkeit wohl bedeutend beeinflusst, aber nicht direct

gereizt werden". GRÜTZNER found no effects of local cooling. The discharge to cooling, so regularly noted by BERNHARD and GRANIT (1946), was probably suppressed by the ether anaesthesia in GRÜTZNER's experiments.

In a later paper GRÜTZNER (1881) intended to analyse the mechanism behind the difference between afferent and efferent fibres with respect to local warming. HERMANN (1871) had already shown that in a muscle a cooled portion was electronegative relative to a warm part. This was known to GRÜTZNER who confirmed his results for nerve but, having missed the effect of cold on the impulse discharge, he, somewhat surprisingly, expected heating to set up a local depolarization capable of generating impulse activity. GRÜTZNER thus followed the same line of thought as BERNHARD and GRANIT (1946) but he did not succeed in demonstrating the expected potential differences between dorsal and ventral roots in response to warming that would have substantiated his theory. The 'cold' potential, by failing to generate a discharge, was another argument against it.

VERZÁR (1911), in reinvestigating the question of 'cold potentials', found (on frogs) that for temperature differences set up below 20° the cooled region was electronegative relative to a warmer region. However, when temperature differences were set up above 20°, he also obtained opposite effects, the warmed portion at times responding negatively. In fact these cases were just as common as the other ones. The negative responses to heating he explained as demarcation potentials due to the high temperature. The temperatures used were, however, never above 38° and thus hardly high enough to kill the nerve. In many cases the negative thermopotential appeared already a few degrees above 20. GALEOTTI and POCELLI (1910) found that at low temperatures the warmed portion of a nerve became electronegative relative to a cooled part but their experimental conditions do not seem to have been altogether satisfactory. The old results are thus of a very conflicting nature which, no doubt, is largely due to the use of frog nerves. As pointed out above, BERNHARD and GRANIT (1946) found mammalian nerves (cat

sciatic) to be in potential equilibrium around body temperature and respond negatively to local cooling or heating (45°).

Different aspects of the problem, falling outside the scope of this paper, were studied by BERNSTEIN (1902), VERZÁR (1911), KOLB (1928), BREMER and TITECA (1930, 1934, 1935, 1946). The results may be summarized in the statement that the demarcation potentials of frog nerves are maximal between $15-20^{\circ}$. BREMER and TITECA (1946) have recently investigated the heat block in frog nerves, immersed in a paraffin bath uniformly heated. The nerves were stimulated with electrical shocks. An interesting finding is the disappearance of the large fibre component of the electroneurogram when the bath was heated.

The effect of temperature has, of course, been analyzed in a large number of papers dealing with the properties of the action potential, chronaxie and accommodation. For summaries, see SCHAEFER (1940) and SCHOEPFLE and ERLANGER (1941). This works need not be summarized here.

Fibres conducting nociceptive impulses.

It will be shown below that stimulation by heat elicits a typical nociceptive reflex. For this reason it is important to review the much debated question as to which fibres conduct pain impulses.

It will be remembered that HEAD and his associates (1905, 1906, 1908) separated the cutaneous sensations into two groups, to which they applied the terms 'protopathic' and 'epicritic'. The protopathic group contains pain and temperature sensations under 22° and over 40° . It is characterised by a peculiar tingling quality and by radiation into other parts than those stimulated. The epicritic group contains light touch and temperature sensations between 22° and 40° . Sensations of the latter kind are all accurately localised. In several important papers RANSON (see RANSON 1921) showed that the protopathic sensations are principally carried in the unmyelinated fibres. He compared the ease with which nociceptive reflexes were elicited from different skin areas with the relative and absolute number of unmyelinated fibres from these areas. Further he showed, confirming HEAD

et al. (1904), that in the regeneration of a nerve the protopathic sensation returns earlier than the epicritic and at the same time as the unmyelinated fibres have grown out. The afferent unmyelinated fibres regenerate at the same time as the vegetative efferent fibres to blood vessels and sweat glands. RANSON further based his assumption on the specific localization of the unmyelinated fibres at the point of entrance into the spinal cord where they pass to the tract of Lissauer and the substantia gelatinosa Rolandi. At the point of entrance into the spinal cord (cat) the unmyelinated fibres have also become differentiated from the myelinated fibres and lie in the lateral section of the root bundles, while the myelinated fibres enter in the medial section.

RANSON and BILLINGSLEY (1916) then proceeded to sever either group of fibres selectively. If the lateral section of the root entrance was severed and thereby the unmyelinated fibres, they did not obtain any nociceptive reflexes to stimulation of this root by a strong faradic current. These reflexes were, on the other hand, still present if only the myelinated fibres were severed by a medial incision in the root.

The unmyelinated fibres ascend ventrolaterally to the tract of Lissauer and the substantia gelatinosa Rolandi (RANSON, 1914). RANSON and HESS (1915) found that the tract of Lissauer and the substantia gelatinosa are closely associated with pain reception and pain conduction and that they likewise transmit the afferent impulses involved in the vasoconstriction reflex due to painful stimulation of the sciatic nerve. The vasomotor reflexes are produced almost exclusively by pain and temperature stimuli. RANSON and HESS believe that the tract of Lissauer and the substantia gelatinosa Rolandi form an organ for the reception and intersegmental conduction of afferent pain impulses. Some impulses from this region, passing over to the spino-thalamic tract, would reach the cortex and find expression as conscious pain, while other impulses, ascending and descending within it, would produce pain reflexes. In their studies on the vasomotor reflex arcs, RANSON and BILLINGSLEY also showed that both pressor and depressor reflexes can be transmitted by the unmyelinated fibres but that their intraspinal tracts are differentiated.

HINSEY and GASSER (1930) found that the thinnest fibres conduct the antidromic vasodilation, released at the periphery by stimulation of the peripheral end of the cut posterior root (STRICKER, 1876) and that these fibres were responsible for the C wave¹ of the electroneurogram. Not until the histological investigations by DUNCAN (1934) could it be definitely stated that the C wave is caused by the whole of the unmyelinated fibre group and by that alone.

BISHOP et al. (1933) and HEINBECKER et al. (1933) in experiments comparing the electroneurogram with the effect of stimulation, could not at first observe any further nociceptive reaction when the stimulation reached the strength at which the C wave was also excited. However, CLARK, HUGHES and GASSER (1935) showed that the unmyelinated fibres of the dorsal roots principally conduct pain impulses. Their conclusions were then confirmed by BISHOP and HEINBECKER (1935), ZOTTERMAN (1936, 1939), GERMANDT and ZOTTERMAN (1946).

Nociceptive impulses are not only conducted by the unmyelinated C fibres. HEINBECKER, BISHOP and O'LEARY (1933) found, on exciting a peripheral nerve of a lightly anaesthetised animal under oscillographic control with shocks progressively increasing in strength, that the nociceptive reflexes became fully developed when the fibres of the δ group were also excited. Various observations by PIÉRON (1930), ADRIAN (1931), HOGG (1935) indicate that sensations of pain may be conducted by thin myelinated nerve fibres. This has also been shown by ZOTTERMAN in the works mentioned (see also ZOTTERMAN 1941 and BISHOP 1946). According to SJÖQVIST (1938) pain impulses are probably conducted by the thin myelinated fibres in the trigeminal nerve of man. GASSER (1943) has reviewed the relevant literature and in his summary of the results states, that the fibres belonging to different modalities must be widely distributed throughout the various fibre sizes and that there seems to be little possibility of associating any one sensation with merely one elevation of the electroneurogram; he states further that it

¹ Terminology according to ERLANGER and GASSER (1937).

seems to be definitely established that pain impulses are carried in both the myelinated and unmyelinated fibres.

However, nobody has been prepared to take up the standpoint that typical nociceptive reflexes can be conducted by the thicker myelinated fibres. Not until the shocks in electrical stimulation have been made sufficiently strong for the thinnest afferent myelinated fibres to become excited, have these reactions occurred and they have markedly increased in strength when, upon further increasing stimulus strength, the unmyelinated fibres also have become involved.

AUTHOR'S INVESTIGATIONS

Technique and Procedure

The experiments have been carried out on adult cats and rabbits. Most cats were decerebrated under ether after ligation of the carotid arteries. Two hours were allowed for removal of the narcotic before the experiments were begun. Some cats were given chloralose intravenously in doses of 0.05—0.06 g/kg. In some experiments cats were used which had received 0.35—0.5 ml Dial Ciba/kg. The rabbits were all anaesthetized with 0.4—0.5 ml/kg Dial Ciba given intravenously.

The blood pressure was measured in the carotid artery, sometimes in the femoral artery, by means of an Hg-manometer writing on smoked paper. In some experiments when it was desired to record the heart frequency a rubber membrane manometer (HÜRTLE) was used. For optical recording of the blood pressure a manometer of the latter type was used. The writing lever was exchanged for a coil moving with the changes in pressure relative to another fixed coil. One coil was fed with a high frequency current (10,000 c.p.s.) the other connected to an amplifier and a cathode ray oscillograph for recording of the blood pressure on a moving film. This system was calibrated by means of an Hg-manometer.

Muscle contractions were only recorded optically. The two coils were shifted to a torsion-wire myograph and the system calibrated by loading the myograph with weights.

Respiration was qualitatively recorded on smoked paper by means of a little respirometer connected to one branch of the tracheal cannula.

The local changes in the temperature of the nerve were set up by means of a thermode, a small lacquered metal container with

a cross-section like a shallow U forming a deep narrow groove for the nerve. Thermodes of different lengths, 2.5, 2.0 and 1.0 cm, have been used but unless otherwise mentioned the 1 cm thermode was the one employed. The temperature of the thermode was regulated by means of circulating water from 2 large containers one filled with hot water the other with ice water. The temperature was measured by means of a thermocouple, the warm junction of which was attached to the thermode the cold junction being immersed in ice water. The temperature was then read on the scale of a Cambridge spot galvanometer of short period. When recording on smoked paper the investigator indicated by means of a signal when the temperature changes started, passed certain values and stopped. The temperature curves were drawn from these markings as seen in the figures, e.g. fig. 1. The thermocouple could also be connected to a "Multiflex" mirror galvanometer (period 1 sec.) reflecting two lights beams to the film, one of them for the lower temperatures the other for the upper range of temperatures (see e.g. fig. 7).

The nociceptive reflex to thermostimulation

If a thermode on the intact sciatic nerve of a decerebrated cat is warmed a strong nociceptive reflex is produced. This agrees with GRÜTZNER'S result (see Historical section). The animal responds as to severe pain. Flexor contraction occurs in the ipsilateral leg and extensor contraction in the opposite one. The body bends ventrally and the head is pulled back towards the trunk. At the maximum of the effect micturition is quite common. Even other involuntary reactions are observable. The motility of the gastric ventricle changes. Both contraction as well as relaxation have been noted. On animals anaesthetized with Dial or chloralose a dilatation of the pupils has been observed. Those animals which have received Dial do not show much of the general motor activity, but those on chloralose respond as the decerebrated ones.

The breathing is greatly affected, but the effects vary from animal to animal. As a rule the amplitude is decreased, even down to apnea; the frequency either increases or slows down. The blood pressure, measured in the carotid artery, rises steeply. The increase can sometimes be greater than 150 mm Hg (fig. 1). In most cases the increase is between 60 and 70 mm Hg. Since prior to decerebration both carotid arteries have been ligated the damping effects of the baro-receptors in the carotid sinus is lacking. Cats anaesthetized with Dial sometimes respond with a decrease in blood pressure to such temperature stimuli as produce pressor effects in decerebrated ones, those anaesthetized with chloralose generally give pressor reactions. Rabbits under Dial regularly give depressor reflexes both to temperature stimuli, high frequency electrical stimulation, as well as to nociceptive stimuli of the skin such as hard pinching with an artery forceps.

Curare, administered intravenously in a dose which produced complete paralysis of the breathing muscles, did not change the

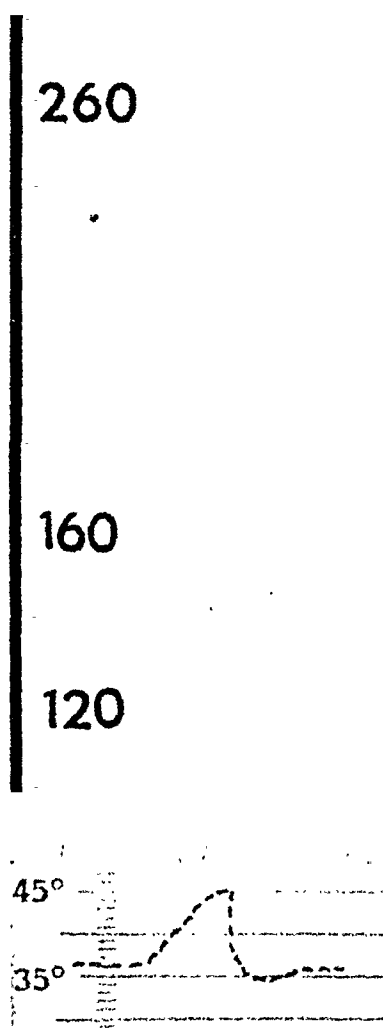


Fig. 1. Cat, decerebrated. Lower tracing: blood pressure (mm Hg). Upper tracing: respiration. Thermode on the intact sciatic. Dotted line: temperature of the thermode (Centigrades). Time in 30 sec.

involuntary reflexes. Therefore the vegetative reactions can not be secondary to the somatic motor activity.

Cooling the thermode on the unsevered sciatic nerve to a temperature approaching 15° hardly ever succeeded in eliciting the nociceptive reflex. Only in 1 of 44 decerebrated cats did local

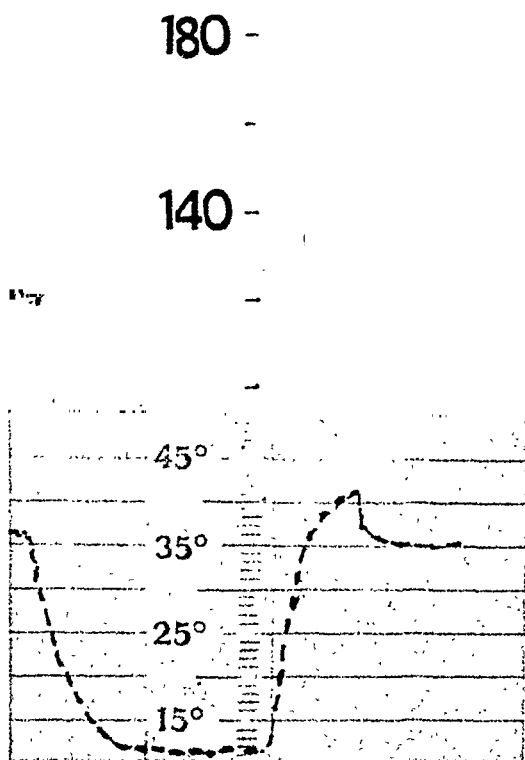


Fig. 2. Cat, decerebrated. Blood pressure (mm Hg). Dotted line: temperature of the thermode. Thermode on the intact sciatic. Time in 30 sec. Note, an unusual effect of cooling.

cooling produce a vasomotor effect, a minor increase in blood pressure of 20 mm Hg (fig. 2). However, in cats on Dial, vasomotor and respiratory reactions are not so seldom elicited by local cooling of the sciatic nerve. These preparations gave either pressor or depressor reflexes. Rabbits on Dial responded to warming of the sciatic with depressor reflexes alone. It was unusual for cooling to produce any vasomotor reflexes in these preparations.

Fig. 3 shows the effect on the blood pressure of local warming of the unsevered sciatic nerve of a cat for different gradients of temperature and for different stimulus durations. In A the ther-

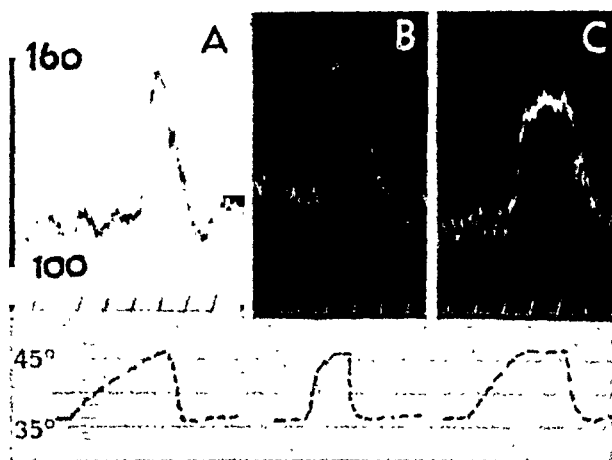


Fig. 3. Cat, decerebrate. Blood pressure (mm Hg). Dotted line: thermode temperature. Thermode on the intact sciatic. Time in 10 sec. For full explanation see text.

mode temperature rises from 36° to 46° in 30 sec. while in B the same temperature increase occurs in 10 sec. The increase in blood pressure starts at practically the same temperature in A and B. The latent period between the instant at which the impulse discharge is set up and that of the increase in the blood pressure is roughly shown in fig. 12. Working with electrical stimulation BERNHARD and SKOGLUND (1942) found a latent period of a few seconds which agrees well with that in fig. 12. If this latent period be included in the comparison between A and B in fig. 3 the impulse generation can be assumed to occur at a few degrees lower temperature in B than in A. As appears from several such comparisons the temperature gradient is therefore probably of importance for the liminal temperature of a blood pressure response. The liminal temperature will be defined as the thermode temperature at that instant when the blood pressure reflex begins. This threshold temperature is also dependent upon the temperature of that portion of the nerve, which is just outside the thermode. Now the sciatic nerve is well protected from cooling even when it lies in the thermode so that in this case the liminal temperature would remain reasonably constant even if the animal itself

were surrounded by air at average room temperature. But actually its body temperature was maintained practically constant by a set of electric bulbs below the operation table. In experiments, carried out under average room temperature, with other nerves which could not be equally well protected from loss of heat, the liminal temperature sank 1° to 3° during 2 to 4 hours. On the other hand, when the animal was placed in a box at a constant temperature of 37° (saturated humidity) the liminal temperature also remained constant during the whole experiment. The liminal temperature varied individually. Of the 59 animals on which I studied blood pressure reactions elicited from somatic peripheral nerves there were only 6 cases in which in the beginning of the experiment the liminal temperature was higher than 45° . In 43 cases this temperature has been between 42° and 44° , and in 6 cases between 40° and 42° . Cats and rabbits have behaved similarly in this respect.

A comparison of a 2.5 cm and a 1.0 cm thermode showed no difference in the liminal temperatures or of the character of the responses.

Severing the nerve distal to the thermode (sufficiently far distal to prevent thermal stimulation of the cut end) did not change the responses, nor could any vasomotor reflexes be elicited from the peripheral end.

The complex pseudo-affective reflex pattern (WOODWORTH and SHERRINGTON, 1904) elicited by local heating of the unsevered sciatic nerve does not digress in character or range from those reactions elicited by relatively high frequency of electrical stimulation of the same nerve (RANSON, 1921, ASHKENAZ, 1939, BERNHARD and SKOGLUND, 1942 and others). It is concluded (see Historical section) that the fibres which mediate the nociceptive reflexes belong to the δ group and the C class. Local heating of the sciatic nerve is, therefore, capable of eliciting impulses in those afferent fibres which belong to the thinnest myelinated or unmyelinated fibre groups. On the other hand, local cooling of this nerve (one exception) had no stimulating effect on these fibres.

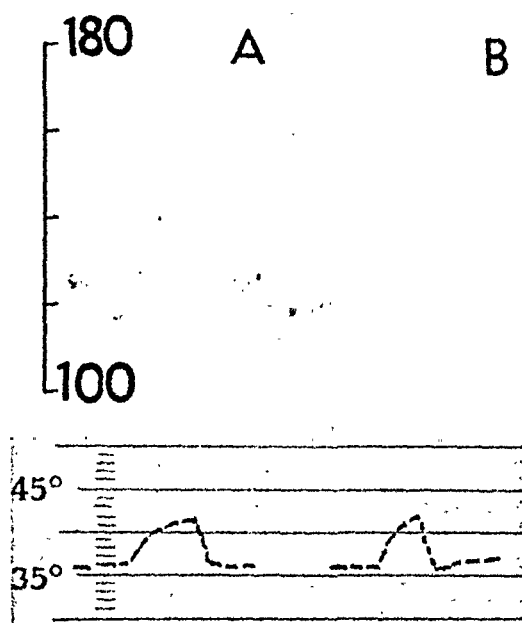


Fig. 4. Cat, decerebrated. Blood pressure (mm Hg). Dotted line: temperature of the thermode. A, before and B, 30 min after intravenous administration of 0.1 mg/kg Gynergen. Thermal stimulation supramaximal. Time in 30 sec.

Effect of ergotamin

ROTHLIN (1923) demonstrated that small doses of ergotamin abolished the effect of stimulating the depressor nerve of the rabbit. WRIGHT (1930) confirmed this (cat) and showed further that ergot in small doses lengthened the latent period of the pressor reflex to strong faradic stimulation of the peroneal nerve. He assumed a central inhibition.

U. S. v. EULER and SCHMITERLÖW (1944) showed that ergotamin tartrate in small doses (0.1 mg/kg) abolishes the baroreceptor reflexes from the carotid sinus. They also showed that the effect must be of central origin since changes in the pressure in the carotid sinus elicited changes in the impulse pattern from Hering's nerve.

The effect of ergotamin was studied on the pressor reflexes elicited by heating of the sciatic in 7 decerebrated cats. The

pressor reflexes were compared before and 30 min. after the intravenous administration of 0.1—0.2 mg/kg ergotamin tartrate.¹ Except in two cases when the average blood pressure had dropped during 30 min. to about 70 mm Hg, the pressor reflexes before and after the administration of ergotamin were practically equal (fig. 4). Thus ergotamin in these small doses did not affect these nociceptive pressor reflexes, indicating the possibility that this substance has a very selective effect upon the vasomotor centre.

However, GERNANDT and ZOTTERMAN (1946) came to the conclusion that the effect of ergotamin even in these small doses partly is to be referred to the periphery on the efferent side.

¹ Sandoz A.-G., Bosel, kindly supplied me with "Gynergen".

Thermostimulation and fibre size

Muscle nerves.

According to RANSON and DAVENPORT (1931) the ratio between unmyelinated and myelinated fibres in the sympatectomized nerve to the vastus medialis is 0.4 to 1, O'LEARY, HEINBECKER and BISHOP (1934) give this ratio as 0.25 to 1. ECCLES and SHERRINGTON (1930) found the ratio between afferent and efferent myelinated fibres to be about 1:1. This indicates that the ratio between unmyelinated and myelinated afferent fibres is between 0.5 and 0.8 to 1. (This ratio in the purely sensory saphenous nerve [after sympatectomy] has been found by RANSON and DAVENPORT, in 2 cases, to be 3.5 and 3.7 to 1.) However, nociceptive impulses are also conducted in the thinner myelinated fibres of the δ group, as shown by O'LEARY, HEINBECKER and BISHOP (1934). This group, according to ECCLES and SHERRINGTON, represents about 25 % of the afferent myelinated fibres (see fig. 5). Thus nearly half the total number of afferent (myelinated and unmyelinated) fibres could be responsible for nociceptive reflexes elicited by stimulation of a muscle nerve.

The effect of thermal stimulation of muscle nerves has been studied in 13 animals.

In fig. 6 (B) is shown the effect on the blood pressure of local heating of the unsevered branch to the vastus medialis of the femoral nerve. A definite pressor response is produced. This effect is, however, decidedly smaller than that which is elicited from the saphenous nerve (A) and from the femoral nerve (C). In all cases supramaximal temperature stimulation has been used and the temperature gradient has been practically the same. In fig. 11 (C) is shown the effect of local heating of the nerve to the medial head of the gastrocnemius muscle. In comparison with a similar response to heating of the sciatic and post. femoral

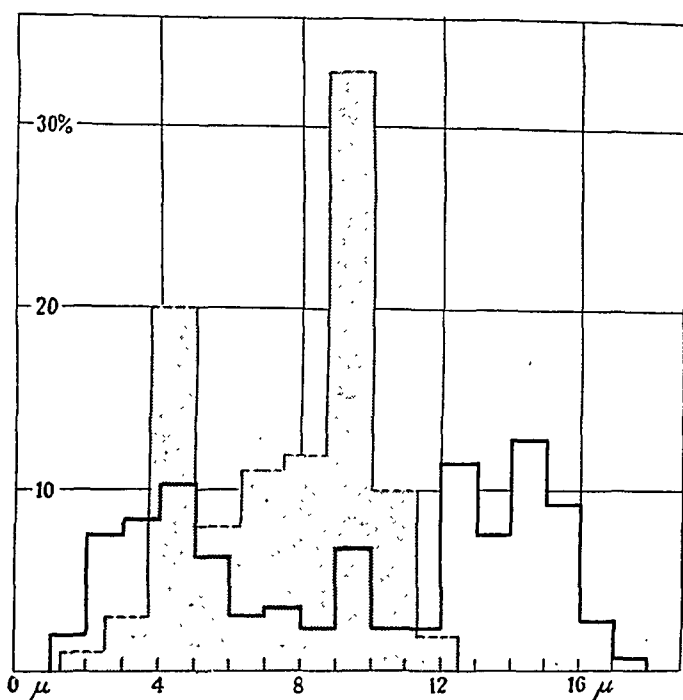


Fig. 5. Diagram of the calibre spectra of the "demotored" nerve of gastroc. med., consisting of 242 afferent fibres (———) and of a dorsal, digital nerve at its proximal end, sampled by 200 fibres (— — —). From ECCLES and SHERRINGTON, *Proc. Roy. Soc., London*, s.B. 106, 326, 1930.

cutaneous nerves (A and B) the effect from the muscle nerve is small. These results are typical.

Local cooling, down to about 15°, of an intact muscle nerve has never elicited any blood pressure reflex.

In order to study the efferent fibres the tendon of the gastrocnemius was attached to a myograph, which recorded isometrically in the manner described on p. 14. The thermode was placed on the nerve to the medial head of the muscle, care being taken to avoid that the muscle itself was stimulated directly by the temperature changes. The dorsal roots L_6 — S_2 and the ventral roots L_7 — S_1 were cut. The peripheral end of one of the cut ventral roots (L_7 or S_1) was divided into filament strands from which the impulses were picked up by silver pin electrodes leading to a capacity coupled amplifier and a cathode ray oscillograph

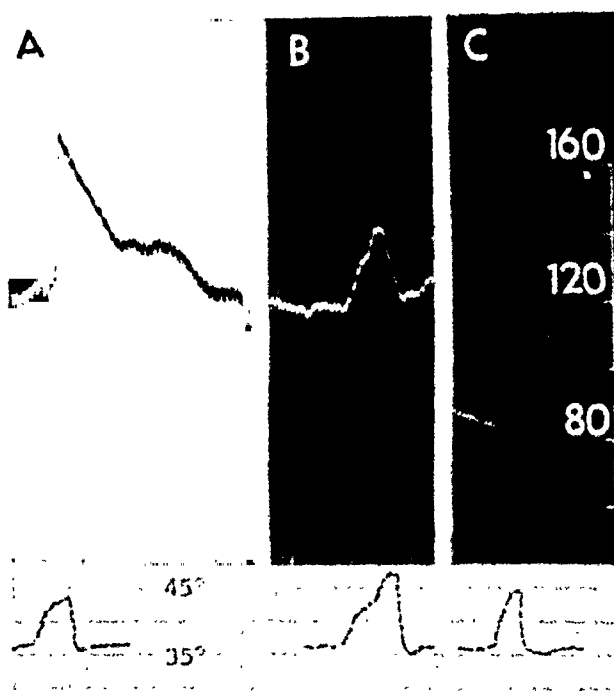


Fig. 6. Cat, decerebrate. Blood pressure (mm Hg). Dotted line: Thermode temperature. Thermode on (A) the intact saphenous nerve, (B) the intact nerve to vastus medialis, (C) the intact femoral nerve. Each stimulus supramaximal. Time in 30 sec.

with a sweep circuit. The frequency of the sweep was generally 1 per 1.2 sec. The action potentials were recorded on the same film as the thermode temperature and the myogram (see Technique and Procedure).

Fig. 7 gives an example of such an experiment. (B) illustrates the muscle tension and spontaneous activity in a ventral filament of S_1 at a temperature slowly falling from 37.4° to 34.8° ; in (A) the temperature has been raised to 46.8° . Heating is seen not to cause any change whatever nor is there activity in the ventral root. That intact fibres in this filament actually passed to the medial head of the gastrocnemius muscle was repeatedly checked by electrical stimulation peripheral to the thermode. On cooling, the muscle contracted more and more and at the same time a discharge appeared in the ventral root filament. (C) shows a

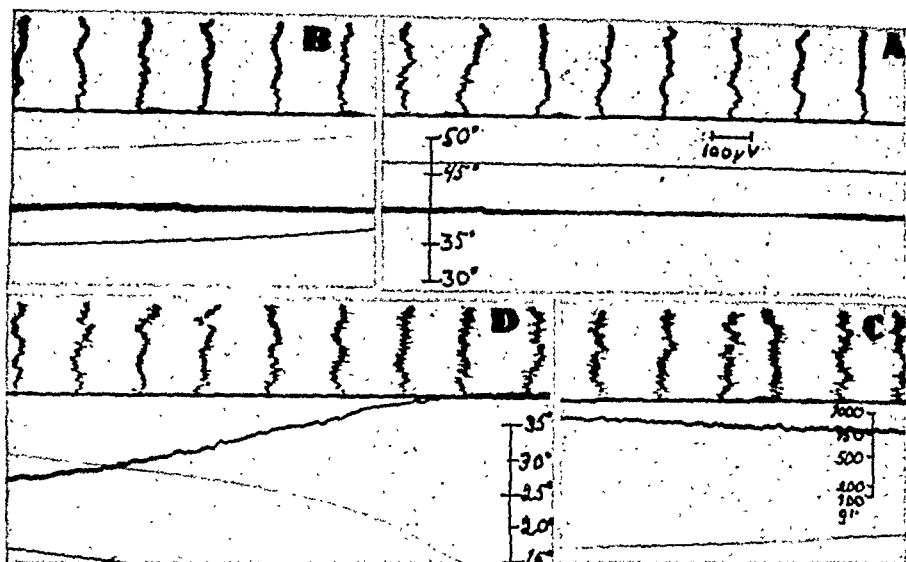


Fig. 7. Cat, decerebrate. Thermode on the intact nerve to the medial head of the gastroc. muscle. Simultaneous records of impulse activity in ventral root filament on vertical sweep, thermode temperature on the two parallel thin lines and tension of gastroc. (thick line) A. Thermode temperature at about 47° . Calibration of the lower temperature line (the uppermost thin one). Muscle tension uninfluenced as is shown by comparison with B. In B the temperature slowly falls from 37.4° to 34.8° . C. during cooling 18.5° to 16.4° . Calibration of tension which now is increased to about 900 g. D. temperature rising from minimum, 14.0° , to 30.3° , muscle tension falling back to normal while impulse activity subsides. Calibration of the upper temperature line. Sweep frequency 1 per 1.2 sec. Sweep time 50 msec. To be read from right to left.

part of the response during cooling. The thermode temperature fell from 18.5° to 16.4° . Finally in (D), the thermode temperature is 14.0° and rises once more. The excitatory effects disappear. This response to local cooling of a muscle nerve is typical and repeatable.

The motor efferent fibres are thick myelinated A fibres. These responded to cooling but never to local heating. In this laboratory LEKSELL (1945) has shown that the thin myelinated efferent fibres to muscles do not elicit measurable contractions.

GRANIT and LUNDBERG (1947) have more closely studied cold and heat stimulation of afferent and efferent fibres in muscle

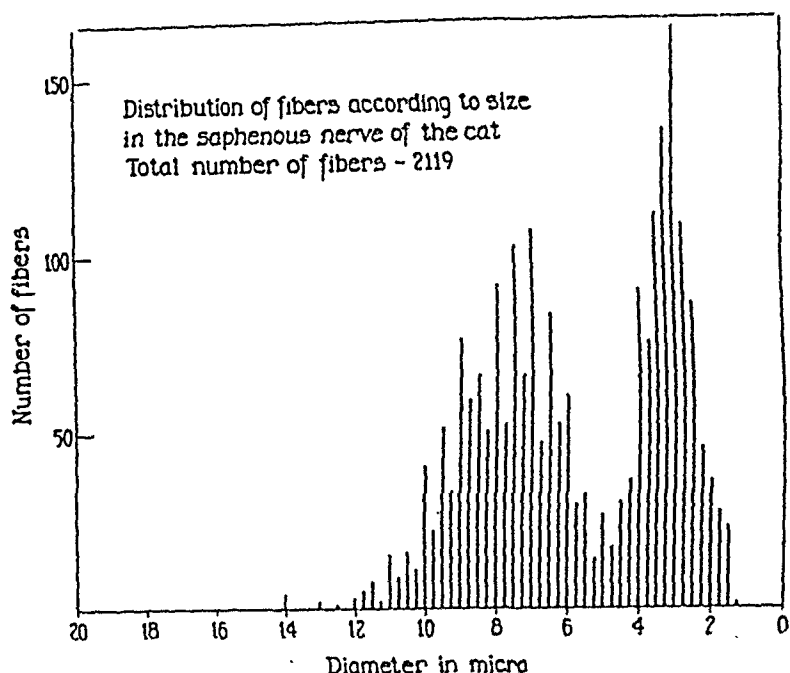


Fig. 8. From GASSER and GRUNDFEST, Amer. J. Physiol. 127, 401, 1939.

nerves and found that an ipsilateral flexor reflex and a crossed extensor reflex are easily produced by heating while proprioceptive large fibre reflexes can be elicited by cooling. Their work should be consulted for details.

Sensory nerves.

RANSON and DAVENPORT (1931) calculated the ratio between unmyelinated and myelinated fibres in the saphenous nerve of the cat and found this to be 3.5—3.7 to 1. They found a similar figure for other sensory nerves supplying skin areas which did not possess a high tactile sensitivity. The thin myelinated δ fibres constitute approximately $1/3$ of the total number of myelinated fibres. The total number of myelinated fibres in the saphenous nerve of the cat is about 2,200 (DOUGLASS, DAVENPORT, HEINBECKER and BISHOP, 1934, GASSER and GRUNDFEST, 1939) (see fig. 8). From these findings the number of unmyelinated fibres

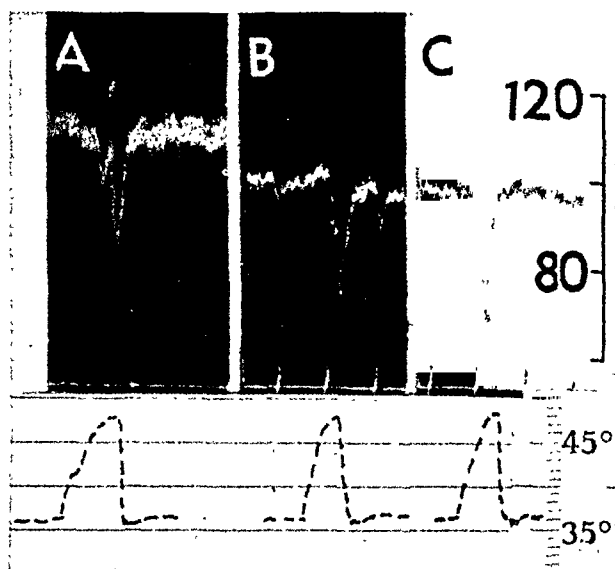


Fig. 9. Rabbit, Dial. Blood pressure (mm Hg). Dotted line: temperature of the thermode. Thermode on (A) the intact saphenous nerve, (B) the intact sural nerve and (C) the intact sciatic. Each stimulus supramaximal. Time in 30 sec.

in this nerve is calculated to be about 8,000 and the δ fibres about 700.

Among nerves distributed entirely to the skin, three, the saphenous, the sural and the posterior femoral cutaneous nerves, have been studied with respect to the effect on the blood pressure elicited by heating and cooling. These experiments have been made on 24 decerebrated cats and rabbits anaesthetized with Dial, mostly on unsevered nerves. A sufficiently long section of the nerve was prepared so that it could be placed in the thermode without stretching. About half of these experiments have been carried out with the animal placed in a box at a constant temperature of 36° and saturated humidity.

The sural nerve was freed in the popliteal space and the thermode well insulated from the surrounding nerves and other tissues. Controls with the thermode in the same position but with the nerve removed showed that this insulation was entirely satisfactory. Local heating of this nerve (in the decerebrated cat) has

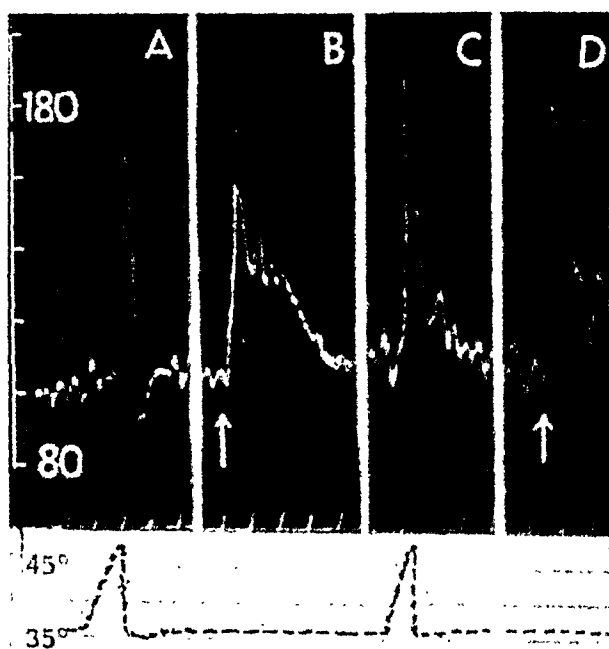


Fig. 10. Cat, decerebrated. Blood pressure (mm Hg). Dotted line: temperature of the thermode. A, heating the intact saphenous nerve (supramaximal stimulation). B, intravenous injection of 5 γ adrenaline. C, heating the intact sciatic (supramaximal stimulation). D, tight ligation of the same nerve. Time in 30 sec.

regularly elicited strong nociceptive reflexes with a considerable increase in blood pressure. On the rabbits (see fig. 9) anaesthetized with Dial, as previously stated, only small motor responses were observed and, as in the heating of the sciatic nerve, a depressor effect was obtained. In fig. 10 (A) is shown the effect of local heating of the saphenous and in (C) of the sciatic nerve in a decerebrated cat. The response from the sural nerve is only insignificantly less than that from the sciatic.

The posterior femoral cutaneous nerve is long and thin, and emerges from the sciatic nerve immediately distal to the branching of the hamstring nerve. When the thermode is placed on this nerve it must be insulated with cotton from the sciatic nerve to avoid stimulation of the latter. Controls were made to show that

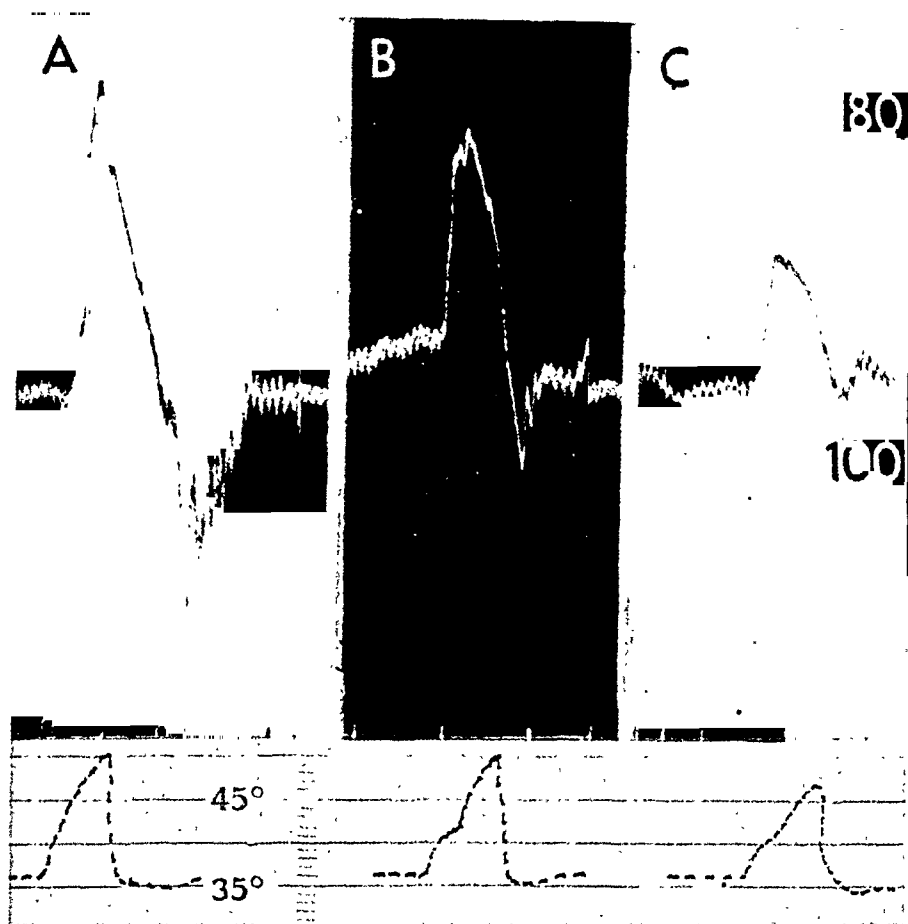


Fig. 11. Cat, decerebrated. Blood pressure (mm Hg). Dotted line: thermode temperature. Thermode on (A) the intact sciatic, (B) the intact post. femoral cutaneous nerve and (C) the intact nerve to the medial head of gastroc. muscle. Each stimulus supramaximal. Time in 30 sec.

this did not occur. Fig. 11 shows a comparison between the effects elicited by local heating from the posterior cutaneous femoral nerve and the sciatic. Here too one is impressed by the small difference in the pressor effects.

In fig. 6 the pressor effects of heating the saphenous and femoral nerves of a decerebrated cat are compared, in fig. 10 those of the saphenous and the sciatic. From these comparisons it appears that a pressor effect elicited by the supramaximal

heating of the saphenous nerve is very nearly equivalent to a corresponding effect from a nerve containing a much greater number of fibres. From figs. 6, 11, and 15 it appears also, as previously mentioned, that there is a very great difference between the effects elicited from sensory and muscle nerves, to the advantage of the sensory nerves. In several experiments the nerve was crushed (fig. 10). This elicited a pressor effect equivalent to that evoked by heat.

From these comparisons it appears that there is a great deal of central overlap to a nociceptive stimulation with consequent occlusion. A relatively limited number of fibres heated sets up maximal pressor effects.

Local cooling has in no case had any vasomotor effect whatsoever. In those cases where the respiration too was recorded, no respiratory reflex could be obtained from local cooling of a sensory nerve in a decerebrated cat. However, on rabbits anaesthetized with Dial a local cooling of a sensory nerve and also of the sciatic nerve has sometimes elicited changes in respiration, usually consisting of an inspiratory suppression and an increase in frequency. Since strong mechanical stimuli and maximal heating elicit equivalent pressor effects, warmth seems to excite most of the fibres which conduct afferent nociceptive impulses. The greater part of these fibres are, as has been demonstrated, unmyelinated. Whether in addition groups of thin myelinated "pain" conducting fibres in these nerves are also excited or not, is impossible to determine on the basis of these experiments.

Action currents in sensory nerves in response to thermostimulation.

The action potentials in thin branches of sensory nerves were recorded in a series of experiments on thermostimulation. In most cases the blood pressure was registered simultaneously.

Technique. 14 cats, most of them decerebrated, a few anaesthetized with chloralose (0.05—0.06 g. per kg. body weight), were used in these experiments. Thin branches of the saphenous and the sural nerves were used. They were layed free for a length of

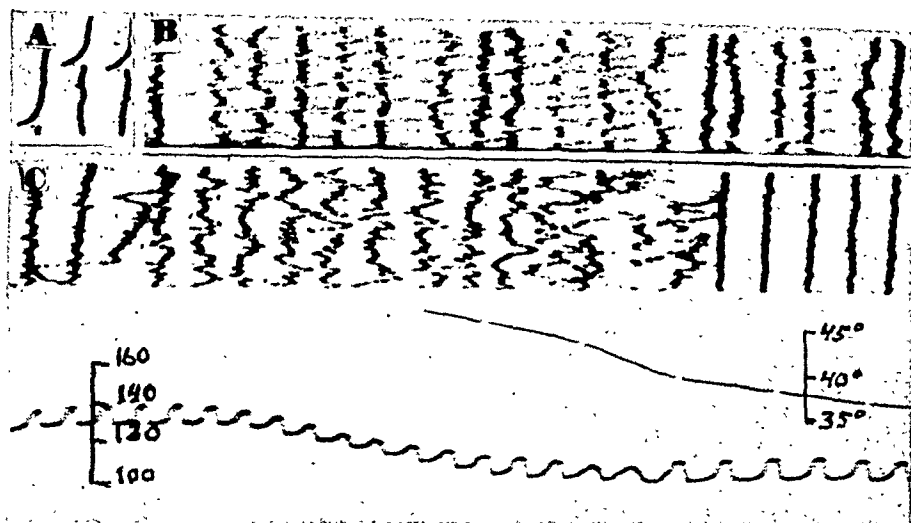


Fig. 12. Cat, decerebrated. Records from a thin branch of the saphenous nerve. A, calibration to 30 μ V. B, light touch of the skin. C, simultaneous records of impulse activity (vertical sweep), thermode temperature (line, interrupted every sec.) and blood pressure (lowest tracing). On first application of heat the animal has moved a little and thrown out amplifier condensers. Later the heat impulses clearly visible. To be read from right to left. Sweep time 25 msec.

about 3 cm but were otherwise intact. All stretching and pinching of the nerve was carefully avoided, and it was kept moist with Ringer solution at body temperature. All these experiments were performed with the animal kept in a shielded, moist chamber at an average temperature of 36°. Silver-silverchloride, sometimes polished silver electrodes were used. The action potentials were led off to the cathode ray oscillograph to be recorded by the sweep at a frequency of 2.4/1 sec. The thermode was placed on the main stem of the nerve, isolated from the tissues. The leg was fastened with drills in order to prevent reflex movements. Temperature and blood pressure were recorded on the same film (fig. 12) (see Technique and Procedure).

In intact branches of sensory nerves there is usually a scattered spontaneous discharge of large single spike potentials, which are believed to originate from large myelinated fibres, and smaller, obviously more slowly transmitted potentials which have a tendency to group themselves into waves. The latter are

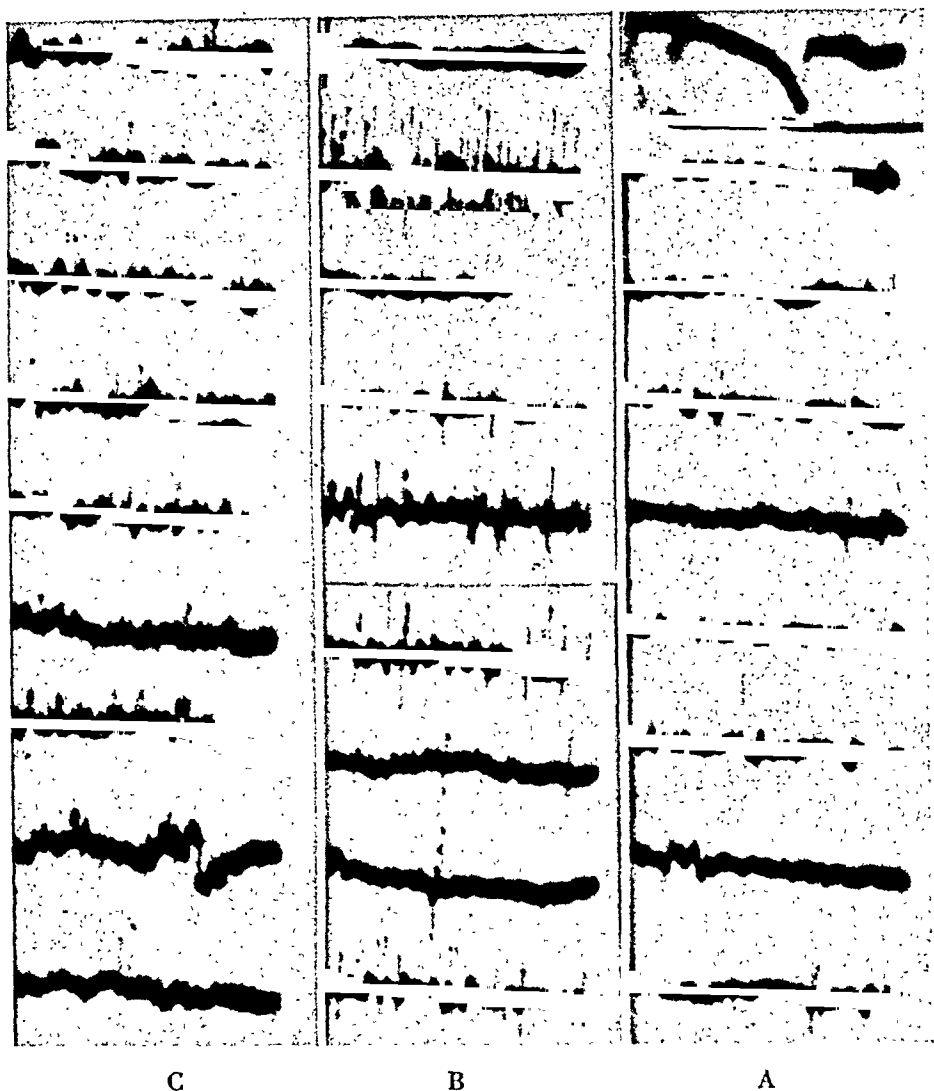


Fig. 13. Cat, decerebrate. Records (to be read upwards) from a thin branch of the sural nerve. Thermode on the main stem of the same nerve. A, spontaneous activity at a thermode temperature of about 37° . Calibration to $30 \mu\text{V}$ in uppermost record. B, touching the skin. Bursts of large, medium and small spike potentials. C, heating the nerve. Marked increase of the small, slowly conducted potentials grouped together in waves. Sweep time 50 msec. Sweep frequency. 2.4 per 1 sec.

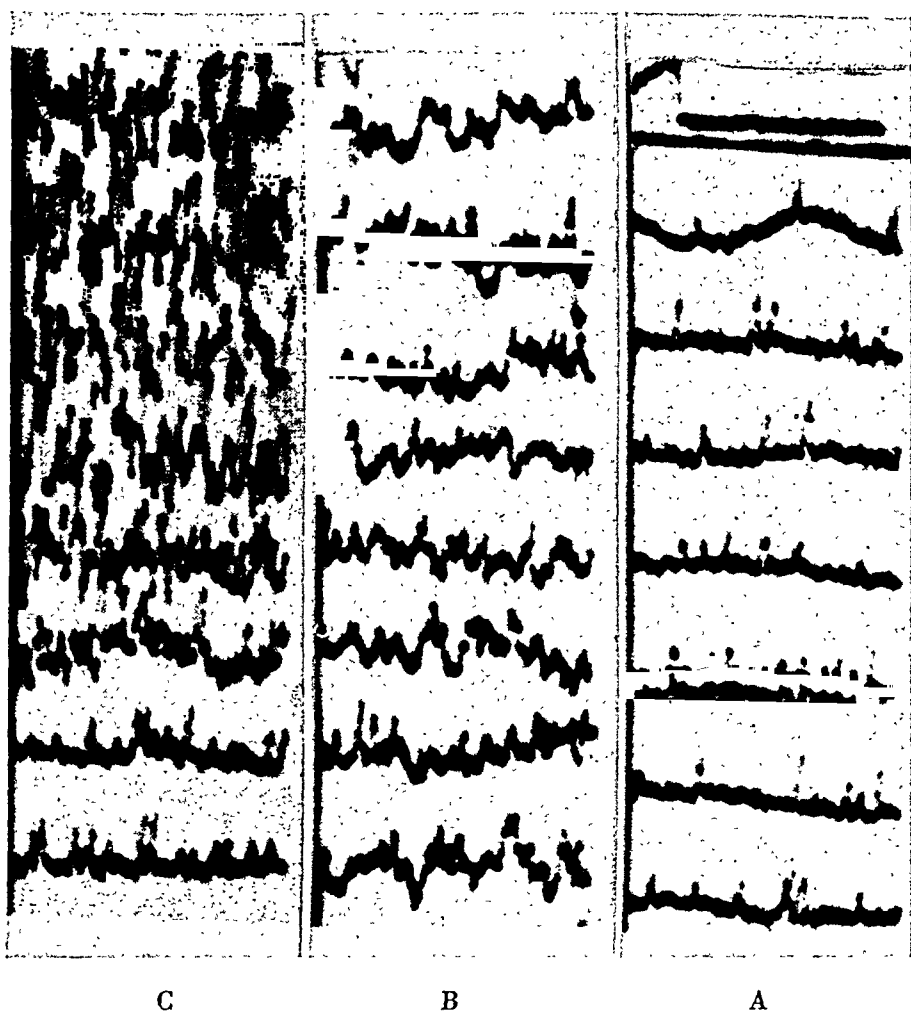


Fig. 14. Cat, decerebrated. Records (to be read upwards) from a thin branch of the saphenous nerve. Thermode on the main stem of the same nerve: A, spontaneous activity at a thermode temperature of about 37° . Calibration to $30 \mu\text{V}$ in uppermost record. B, heating the nerve. Marked increase of the small, slowly conducted potentials grouped together in waves. C, thermode temperature falling from 30° to 19° . Larger, not separated spike potentials appear at about 26° . Sweep time 25 msec. Sweep frequency 2.4 per 1 sec.

believed to originate from the slowest section of the δ group and from fibres belonging to the C class (ZOTTERMAN, 1933, 1936, 1939).

When the hairs on the skin area to which the nerve branch is distributed are touched lightly a discharge of large spike potentials occurs and even the smaller potentials increase in frequency (figs. 12 and 13), as pointed out by ZOTTERMAN (1939) in a valuable analysis of touch, pain and tickling.

If the thermode is heated, small potentials occur which seem to interfere rather much with each other and create irregular waves, in the manner described as typical for the slowest conducted potentials deriving from the thinnest fibres in the δ group and from the C fibres. The large spike potentials have not increased in number (figs. 12, 13, and 14). A few seconds after the first appearance of these potential waves the blood pressure begins to rise (fig. 12).

If, instead, the thermode is rapidly cooled the large spike potentials increase greatly in number. If the nerve branch is not sufficiently thin (fig. 14) it can not be determined whether the small spike potentials increase in frequency or not in response to cooling, but in no such case was the discharge to cooling followed by any blood pressure rise.

In one case, when the nerve was severed central to the thermode, it was observed that a spontaneous discharge of large spike potentials disappeared on stimulation by heat but reappeared upon return to normal temperature and increased on cooling.

Phrenic nerve.

In fig. 15 (A) is shown the pressor effect produced by local heating of the intact phrenic nerve of a decerebrated cat. In the 6 experiments where this nerve has been studied such pressor effects have all been somewhat smaller and varied between 10 and 18 mm Hg. (B) in the same figure shows the pressor effect to heating the nerve to the medial head of the gastrocnemius muscle of the same animal. This effect is much greater than that

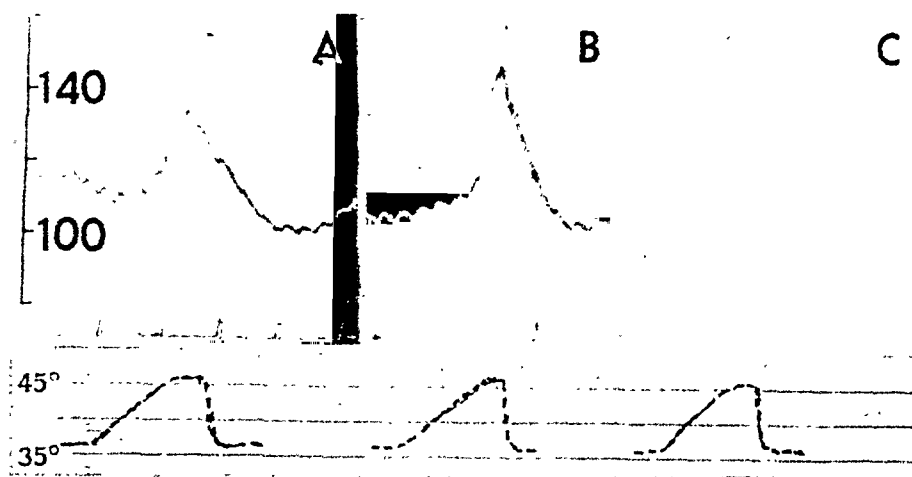


Fig. 15. Cat, decerebrated. Blood pressure (mm Hg). Dotted line: thermode temperature. Thermode on (A) the intact phrenic nerve in the neck, (B) the intact nerve to the medial head of gastroc. muscle and (C) the intact sural nerve. Each stimulus supramaximal. Time in 10 sec.

from the phrenic nerve. In 2 cases a hard pinching of the nerve with an artery forceps resulted in a pressor effect of 15 respectively 18 mm Hg. that is equivalent to the effects of heat stimulation.

GASSER and ERLANGER (1927) came to the conclusion that the phrenic nerve is lacking in the smaller variety of the A-class fibres. DOUGLASS, DAVENPORT, HEINBECKER and BISHOP (1934) found that the phrenic nerve of the cat contains a very small number of unmyelinated fibres (7 unmyelinated of 755 myelinated). They could not show any C wave in the action potential from this nerve. GERNANDT (1946) recorded impulses from thin filaments of the peripheral end of the phrenic nerve of cats and excited the abdominal surface with different noxious stimuli such as, pinching, heat above 40° and chemicals. He never obtained any C potentials, only small potentials. There are a large number of papers by different authors dealing with the conduction of pain impulses in the phrenic nerve of man (FELIX, 1922, MORLEY, 1931, WOOLHARD, ROBERTS and CARMICHAEL, 1932, and others). Nociceptive reflexes elicited from the central end of the severed

phrenic nerve have also been studied amongst others by GREENE (1935), THORNTON (1937), LITTLE and McSWINEY (1938), HINSEY and PHILLIPS (1940) and GRANDJEAN (1943).

HINSEY, HARE and PHILLIPS (1939) showed by means of degeneration experiments that the afferent fibres constitute about 10 % of the total number of myelinated fibres in the phrenic. In 2 of 3 nerves they found that the relation between the unmyelinated and myelinated afferent fibres was 3 to 1 while this ratio in the third nerve was 1 to 1. One of these nerves had been studied by GRUNDFEST with respect to its action potentials. He found that the most rapid fibres had a conduction velocity of 63 m/sec., and that there was a slow wave, made up of potentials from fibres in which the conduction velocity was about 20 m/sec. He found no C spike. These statements by GRUNDFEST support the electro-physiological data obtained by DOUGLASS, DAVENPORT, HEINBECKER and BISHOP on the phrenic nerve of the cat. ONO (1934) gives the relation between unmyelinated and myelinated fibres in the phrenic nerve as 0.18 to 1, and TATESI (1939) as 0.25 to 1. However, these authors have not, as the others, calculated this ratio after degeneration of the unmyelinated sympathetic fibres. The total number of myelinated fibres is estimated by the different authors to be between 600 and 800. It is thus possible to estimate roughly the number of fibres which can conduct nociceptive impulses to about 150—300. This is about 1/3—2/3 the number to be expected in the nerves to the vastus medialis and to the medial head of the gastrocnemius muscle. A comparison between A and B in figure 15 shows roughly such a ratio between the pressor affects from the phrenic and medial gastrocnemius nerve.

Recurrent nerve.

This nerve carries motor fibres to the larynx and sensory fibres to the oesophagus and the trachea and also includes a bundle of sympathetic fibres. MOLHANT (1910) and CHASE and RANSON (1914) have come to the same result through histological

studies of the recurrent nerve. The nerve as it enters the larynx is made up entirely of large myelinated fibres. At its origin this nerve is composed of 2 quite sharply defined areas: one contains the bundle of large myelinated fibres mentioned above, the other contains small and medium-sized myelinated fibres. The latter are distributed in the cardiac, oesophageal and tracheal branches. There is also a third section formed of sympathetic unmyelinated fibres. Their experiments were carried out mainly on dogs but some also on cats and rabbits. TOMONAGA (1939) found that in cats a group of small myelinated fibres continue to the larynx. Electrical stimulation of the recurrent nerve (BECHTEREW, 1908 and others) produces a retardation of the breathing.

Local heating of the recurrent nerve as close to the entrance into the larynx as possible has only in a few cases elicited any vasomotor reaction, and in these cases the reaction has been very small. However, if the thermode was placed as near as possible to the exit of the nerve from the thorax a small vasomotor reaction was regularly elicited, usually an insignificant pressor effect of 6—12 mm Hg. In one case crushing of the nerve elicited a pressor effect of 12 mm Hg. Local heating had in this case produced the same pressor effect. In 2 experiments a depressor effect of 18 mm Hg. was obtained by local heating of the recurrent nerve at this level. Local cooling near the entrance into the larynx never gave any vasomotor reaction but at a more proximal position small depressor effects could sometimes be obtained. Both heating and cooling at the proximal level of the recurrent nerve elicit respiratory reactions such as a reduction in frequency and amplitude. Effects from cooling have occurred at a thermode temperature between 25° and 20°. Effects of local cooling on the respiration have been followed in some cases by a similar small effect appearing immediately after the temperature has begun to return to the normal. This is probably not a true heat effect (cf. p. 41), because it occurs already at thermode temperatures between 15° and 20°. Moreover it does not have the characteristics of a heat effect. It is, for instance, never followed by any vasomotor reaction. More pronounced "off" effects could

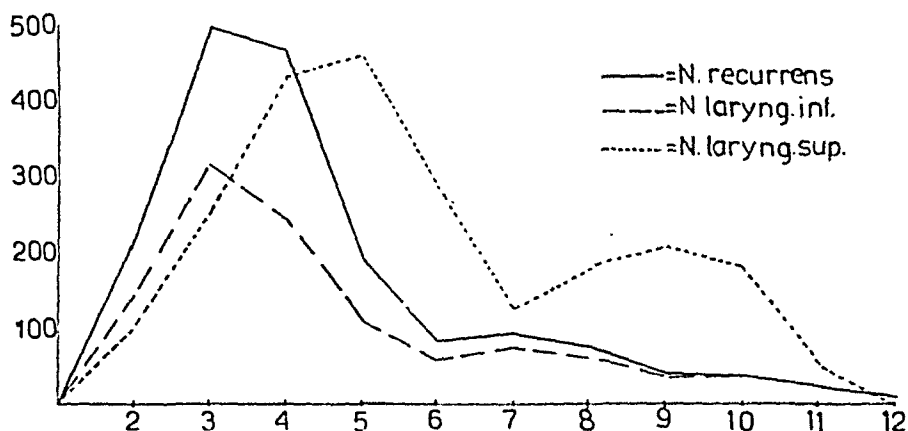


Fig. 16. Diagram of calibre spectra drawn from two figures of TOMONAGA, 1939 (ordinate: number of fibres, absciss: calibre in μ).

be elicited by cooling of the superior laryngeal nerve (see below). GRANIT and LUNDBERG (1947) have also described such "off" effects due to removal of the cold stimulus in large fibres.

Superior laryngeal nerve.

This nerve emerges from the lower pole of the nodose ganglion of the vagus nerve, and carries motor fibres to the crico-thyroid muscle in the larynx and sensory fibres from the laryngeal mucosa, it also carries sympathetic fibres from the superior cervical ganglion. This nerve has been studied histologically by CHASE and RANSON (1914). They found that it contains large, medium and small myelinated fibres, the small and medium fibres predominating, unmyelinated fibres are also present in large numbers. TOMONAGA (1939) shows that the small myelinated fibres are fewer in comparison with the medium-sized ones in the cat. The frequency maxima occur at 5 μ and 10 μ , the comparative maxima for the recurrent nerve occur at 3 μ and 7 μ respectively (fig. 16).

Because this nerve is only about 2 cm long particular care must be taken when placing the thermode on the nerve. It must

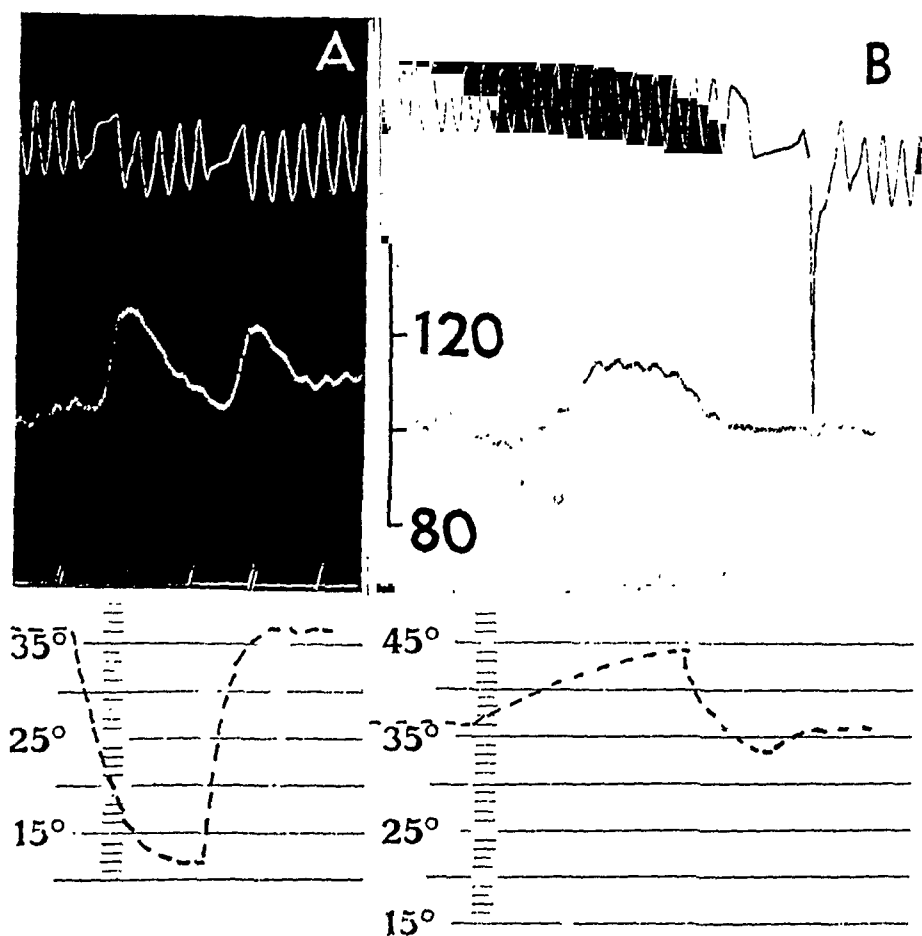


Fig. 17. Cat, decerebrated. Upper tracing: respiration. Lower tracing: blood pressure. Dotted line: thermode temperature. Thermode on the intact superior laryngeal nerve. A, cooling and B, heating. Time in 10 sec.

be insulated with cotton from the main stem of the vagus nerve to avoid any effects of stimulation from this source, also similarly insulated from the larynx itself so that the nerve endings in the laryngeal mucosa are not influenced by the temperature changes. Controls were made in all cases with the thermode practically in the same position but with the nerve outside it. All these controls proved satisfactory.

Cooling to 15° regularly gave a momentary and often complete depression of the breathing independent of whether the stimula-

tion occurred under inspiration or expiration. In most cases this effect has been followed by a vasomotor reaction, a pressor effect of 10—20 mm Hg. As with cold stimulation of the recurrent nerve an "off" effect is obtained shortly after the temperature begins to rise again towards 36°. Even in these cases the reaction appears so quickly after the turning point (fig. 17) that it cannot be confused with a true heat effect appearing earlier because of the previous cooling. Besides, even in these cases the "off" effect is a typical effect of cold as is most clearly seen in fig 17. Heating does not in any case produce respiratory reactions of this type. Heating elicited in all cases a pressor effect of 15—30 mm Hg. In 2 cases heating resulted in a respiration effect similar in character to that from the spinal nerve, i.e. a change in frequency and a decrease in amplitude. After heating, the return of the thermode temperature (it often fell as low as 35° or 34°) always produced an effect on the breathing of the same type as with cold stimulation. It cannot therefore be interpreted as an "off" effect after heat excitation, but must be explained as a cold effect, which, because of the previous heating, is elicited at a higher temperature than otherwise. GRANIT and LUNDBERG (1947) have also described "off" effects from cold stimulation but have never observed "off" effects from heat stimulation.

The superior laryngeal nerve is described (BECHTEREW, 1908) as the specific inhibitory nerve for the movements of respiration. RIJLANT (1942) has studied these inhibitory reflexes in the cat. Single shock stimuli were used and the effect in the form of action currents from the motor respiratory nerves was studied with the aid of a cathode ray oscillograph. Every shock released after a latent period of 6—8 msec. a short and for the most part total inhibition of the inspiratory nerves. ROGER (1917) has shown that the inhibitory effects on respiration due to stimulation of the laryngeal mucosa are followed by marked cardiac and vasomotor reflexes. The different effects obtained by heating and cooling the superior laryngeal nerve indicates that different fibres are excited in the two cases. As appears from above the fibres are divided into 2 large main groups namely, the unmyelinated and the myelinated ones with a maximum at about 5 μ ,

the maximum at $10\ \mu$ being probably due to the motor fibres and the afferent fibres from the muscle spindles (see fig. 16). The latent period, as found by RIJLANT, for the inhibitory effect of stimulation of the superior laryngeal nerve does not in any way correspond to the conduction velocity for unmyelinated fibres which lies between 0.5 and 2 m/sec. This means that local cooling of this nerve excites the myelinated fibres with a higher conduction velocity, while heating probably mainly excited the thinnest fibres with a low conduction velocity.

Depressor nerve:

The depressor nerve in rabbits runs separate from the vagus and the sympathetic trunk in the neck and first joins with the vagus at the branching of the superior laryngeal nerve. According to HEINBECKER and O'LEARY (1933), and DOUGLASS, DAVENPORT, HEINBECKER and BISHOP (1934) the depressor nerve of the rabbit contains afferent fibres of $6\text{--}3\ \mu$ which give rise to a large δ elevation in the action potential, and in addition unmyelinated sympathetic efferent fibres. GERNANDT (1946) indicates the possibility of efferent vagus fibres as well as afferent spinal fibres, which pass the stellate ganglion and run to the posterior roots.

U. S. v. EULER, LILJESTRAND and ZOTTERMAN (1941) found in the carotid sinus nerve two types of baroreceptive impulses, one large and one small type. In view of the small number of large pressure impulses as compared with the small ones, they considered the latter to be of greater importance for the sinus baroreceptor reflexes. There is good reason to believe that a similar state of affairs prevails in the depressor nerve.

The depressor nerve has been studied on 9 rabbits anaesthetized with Dial. It was freed in the neck, ligated and severed as far distally as possible. The central stump was placed in the thermode and protruded about 2 cm outside it, in order to prevent thermal stimulation at the cut end. Stimulating electrodes were placed on the nerve peripheral to the thermode. Precautions were taken to avoid the spreading of the thermal and electrical

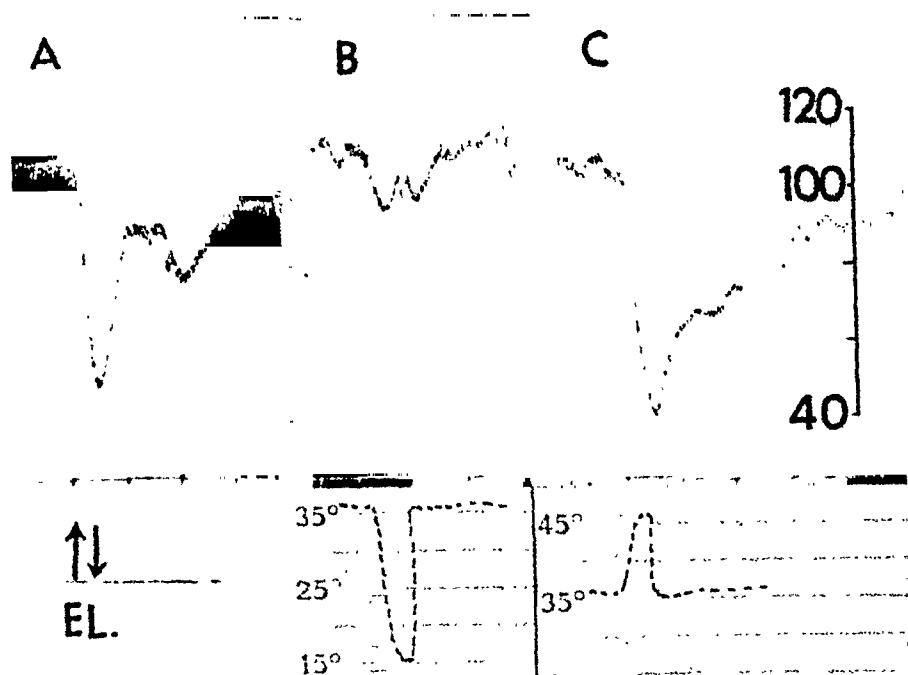


Fig. 18. Rabbit, Dial. Blood pressure (mm Hg). Dotted line: thermode temperature. Thermode and stimulating electrodes on the central end of the severed, right depressor nerve (the left depressor cut and both carotides ligated). A, electrical stimulation, B, cooling and C heating. Time in 30 sec.

stimuli to the surrounding tissues. The nerve was continuously moistened with Ringer solution at body temperature.

In most cases local heating of the central stump of the severed depressor nerve elicited a marked decrease in blood pressure which in many cases was equivalent to that caused by a maximal electrical stimulus (fig. 18). In one case no effect was obtained by heating whereas cooling gave a marked effect. In most cases an effect was obtained from both heat and cold stimuli. The cold effects were, however, never so marked as with maximal electrical stimulation. These results can be explained hypothetically by assuming that local cooling selectively excites the larger fibres (corresponding to the large impulses of v. EULER, LILJESTRAND and ZOTTERMAN) and that local heating selectively excites the thinner ones (corresponding to the smaller spikes). Thus the relatively few large fibres would be excited by cold with a

relatively small depressor effect as result, and the relatively small fibres would be excited by heat with a comparatively large depressor effect as result. The large fibres are as a rule less sensitive to drying and stretching than the thinner fibres. This might explain why in one case only a cold effect and no heat effect was obtained.

Discussion.

The experiments described have shown that local heating of a peripheral somatic nerve elicits nociceptive reflexes with, in general, a marked vasomotor reaction equivalent to the one set up by strong mechanical stimulation of the nerve. These mechanical stimuli, tight ligation or hard pinching with an artery forceps, can be assumed to have been maximal for all fibres. A comparison of the thermally activated vasomotor effects in different large mixed and sensory nerves, has shown that the reflex responses are not proportional to the number of "pain" fibres, unless this number is small as *e. g.* in muscle nerves. Thus, for maximal blood pressure responses a relatively modest number of small afferents seems to be sufficient. The central overlap in the neurone pool must therefore be considerable. A maximal stimulus can in the large nerve stems and in the sensory nerves elicit a pressor effect equal to the effect of heating. This means that the thermal stimulus has given a maximal effect, even if it has left out some of the fibres mediating pressor reflexes. Consider, for instance, the saphenous nerve. The ratio between the number of afferent fibres in the δ group and those in the C class can be calculated (RANSON *et al.*, see p. 27) to be about 1 to 10. In view of the great number of C fibres the effect of a maximal nociceptive stimulus would therefore probably be the same whether the δ fibres are involved in the stimulation or not. On the other hand, the effect of stimulating only the δ fibres is much less than the effect from both the δ group and the C class, as shown by CLARK, HUGHES and GASSER (1935).

These studies of the vasomotor reflexes elicited by local heating of large mixed and sensory nerves have thus shown that the

afferent fibres belonging to the C fibre class are excitable by this mode of thermostimulation and that in general no "pain" fibres, either of the C class nor of the δ group, can be stimulated by local cooling.

In muscle nerves the number of unmyelinated fibres is much smaller. The ratio between unmyelinated and thin myelinated fibres is also much lower in these nerves and can be estimated to be about 3 to 1, using data from RANSON et al. and from ECCLES and SHERRINGTON (see p. 23). As to these nerves it can be assumed that a stimulation of the C group alone would elicit a markedly smaller effect than a stimulation which includes all fibres conducting nociceptive impulses. Now the experiments on muscle nerves also showed that equivalent pressor effects were elicited by strong mechanical stimulation and by local heating. This indicates that heating can stimulate the δ afferents as well as the C afferents.

The presence of afferent δ fibres in the phrenic nerve is well established both by histological and neurophysiological experiments. There is not the same certainty with regard to C fibres. It is doubtful whether in this nerve there are a sufficient number of them to elicit a recordable effect. Severe mechanical stimulation of the phrenic nerve elicited the same pressor effect as heating, which again supports the conclusion that local heating sets up impulses in the δ fibres.

Local cooling of a muscle nerve has been shown to produce a contraction of the muscle, due to stimulation of the thick myelinated motor fibres. Hence, such fibres are susceptible to cooling. Heating has been shown to have no motor effects.

The results obtained from the laryngeal nerves, especially from the superior one, demonstrate that local cooling does not only excite the thick *efferent* fibres. The cooling of those nerves elicited a maximal inhibition of the respiratory movements and in addition a vasomotor reflex. Most kinds of stimuli applied to the mucosa of the larynx elicit a similar reflex (BECHTEREW, 1908, ROGER, 1917 and others). The short latent period found by RIJLANT (1942) proves this inhibitory reflex to have been initiated by impulses conducted in myelinated fibres. This, of

course, is in full agreement with the fact that heat could not elicit inhibition of the respiration but only succeeded in evoking a pure pressor reflex. As the 10 μ group is considered mainly to contain efferent and afferent fibres for the crico-thyroid muscles, the large group of medium sized myelinated fibres (see fig. 16) must be regarded as responsible for the inhibitory reflex. Such fibres must therefore be excitable by local cooling in order to explain my results. Studies of the action currents in the sensory nerves showed conclusively that local cooling excites fibres which are thicker than the δ fibres.

From the fact that local heating is incapable of exciting the motor fibres GRÜTZNER concluded that local heating differentiated between afferent and efferent fibres. However, thermostimulation of the superior laryngeal nerves and the analysis of the action currents in the sensory nerves showed clearly that the thick afferent fibres are not excited by local heating. The sensitivity of the unmyelinated and thin myelinated *efferent vegetative* nerve fibres to thermic stimulation will be discussed in the following section of this paper.

The sensitivity of the depressor nerve to both heating and cooling is easily explained by the fact that it contains fibres of different size, as pointed out above (p. 42). However, an overlapping of the effects of cooling and heating in the same fibres cannot on the present evidence be denied. It must, however, be remembered that the depressor nerve has been studied on animals anaesthetized with Dial. In these animals, as mentioned before, the thinner fibres sometimes tend to respond to cooling. In this they differ from those of decerebrated animals.

It is well known that cooling as well as heating of the skin produces pain and vasomotor reactions (see ZOTTERMAN, 1936, 1939, LEWIS, 1942, EKSTRÖM, LUNDGREN and SCHMITERLÖW, 1943 and WOLF and HARDY, 1943). However, it is impossible to compare these results with those from stimulation of the nerve itself. Thus, e.g. adequate stimulation of cold receptors sets up impulses in the thin fibres connected to them, whereas cooling of the nerve stem itself has been shown to excite large fibres.

Thermostimulation of visceral nerves

Anatomical.

The visceral nerves contain vegetative efferent nerve fibres deriving from the cranio-sacral and the thoraco-lumbar outflow as well as afferent fibres of different size but with the same properties as corresponding fibres in the somatic nerves (BISHOP and HEINBECKER, 1930, HEINBECKER and O'LEARY, 1933). The vagus contains also somatic motor fibres to the muscles in the larynx but the branches of the vagus which carry these do not differ very much from somatic nerves and the results from them have already been described. A very extensive literature deals with visceral sensations, afferent visceral nerve fibres and their relationship to the adjoining efferent fibres as well as the origin of their cells (see HINSEY, 1935, LEWIS, 1942, and "Pain", 1943). This is not the place for a review of this literature to which during 1946 GERNANDT and ZOTTERMAN, DOWNMAN and McSWINEY have contributed and, in 1947, SJÖSTRAND and C. v. EULER. It shows, to sum it up briefly, that the internal organs are supplied with sensory nerve endings and that impulses run from these structures mainly over the vagus nerve to the medulla oblongata and over the thoracic, splanchnic, hypogastric and pelvic nerves to enter the spinal cord through the posterior roots. The cell bodies for these nerve fibres probably all lie in the nodose ganglion respectively the posterior root ganglia and there is no difference in the histological and physiological properties of visceral and somatic sensory nerve fibres. The axons, which carry pain sensations and most of those which mediate vasomotor reflexes, are small, poorly myelinated or unmyelinated and their conduction velocity is slow.

The vegetative efferent nerve fibres are thin myelinated fibres of the B class (BISHOP and HEINBECKER, 1930; then labelled B₂; by B₁ at that time was meant the present δ) and unmyelinated fibres of the C class, the pre-ganglionic fibres belonging prefer-

ably to the B class and the post-ganglionic ones preferably to the C class. BISHOP and HEINBECKER showed also that the B elevation was specific for the autonomic efferents.

According to CHASE and RANSON (1914), RANSON, FOLEY and ALPERT (1933) and FOLEY and DuBOIS (1937), the vagus nerve below the jugular ganglion contains, for the most part, unmyelinated fibres of which the majority are afferents. The last named authors state that the efferent fibres constitute between 20 % and 35 % of the total number of fibres, and that of these, between 48 % and 71 % were myelinated, and the remainder unmyelinated. Of the total number of afferent fibres they estimated that only 10—20 % were myelinated.

HEINBECKER and O'LEARY (1933) showed that the efferent fibres to the heart belong both to the B and C classes. In that paper they also found that the efferent fibres to the stomach and intestine were all unmyelinated. They further maintained that the vagus nerve, at its passage through the diaphragm, almost exclusively contained unmyelinated fibres, confirming CHASE and RANSON (1914), and that at this level it was a practically pure motor nerve. GOLDBERG (1931) found, however, that the vomiting reflex from a distended pyloric pouch was served on its afferent side entirely by the vagus nerve. IRVING and McSWINEY (1935) used the pupil dilatation test on afferent impulses and stimulated the upper duodenum and pylorus by distention. They found that the afferent impulses were transmitted both by the vagus and the splanchnic nerves.

RANSON and BILLINGSLEY (1918) have by degeneration experiments shown that the white rami, the sympathetic trunk and the great splanchnic nerve contain a large portion of afferent unmyelinated fibres and also efferents of all sizes. The efferent part consists of small myelinated and unmyelinated fibres. GERNANDT and ZOTTERMAN (1946) found that, with mechanical stimulation of the intestine, action potentials of δ and C character could be led off from the thin perivascular nerves of the mesentery. SJÖSTRAND and C. v. EULER (1947) found that visceromotor reflexes could be elicited by noxious stimuli of the intestinal wall.

These visceral nerves thus give us a chance of comparing in

the same nerve the thermal sensitivities of thin afferent fibres and vegetative efferent fibres of approximately the same size. The following results contain such a comparison between, on the one hand, reflex vasomotor reactions due to afferent stimuli and, on the other, reactions due to stimulation of the efferent vegetative fibres. A comparison has also been made between the effects of thermal and electrical stimuli.

Vagus nerve.

Technique. Experiments on the vagus nerve in the thorax and the splanchnic nerve were carried out on 7 decerebrated cats. The blood pressure in the carotid artery was measured in the usual way with an Hg manometer or a rubber membrane manometer. To record the movements of the stomach a tube was introduced through a cervical oesophagotomy after ligation of the pylorus which was reached through a small abdominal incision. The stomach was then washed out with warm saline and the tube connected to a piston recorder, the stomach and about half the tube being filled with saline (BROWN and McSWINEY, 1932). The vagus and the great splanchnic nerves were exposed by removal of the 4th to 12th ribs after ligation of the intercostal vessels, a Starling pump being used to maintain sufficient respiration. The diaphragm was cut at the passage of the great splanchnic nerve. The vagus was freed just above the branches to the lung and, also, just above the diaphragm, and the great splanchnic at its passage through the diaphragm. This was done for a sufficiently long section so that without stretching the nerve could be placed intact in the thermode. Stimulating electrodes had been fastened to the thermode, one on each side, and connected to a thyatron stimulator.

These experiments were performed in a box maintaining high temperature and humidity. The vagus nerve was also stimulated in the neck (thermally and electrically), and for this purpose it was freed from the cervical sympathetic trunk. The vagus nerve in the neck was also investigated in rabbits anaesthetized with Dial. In every experiment care was exercised so as to avoid

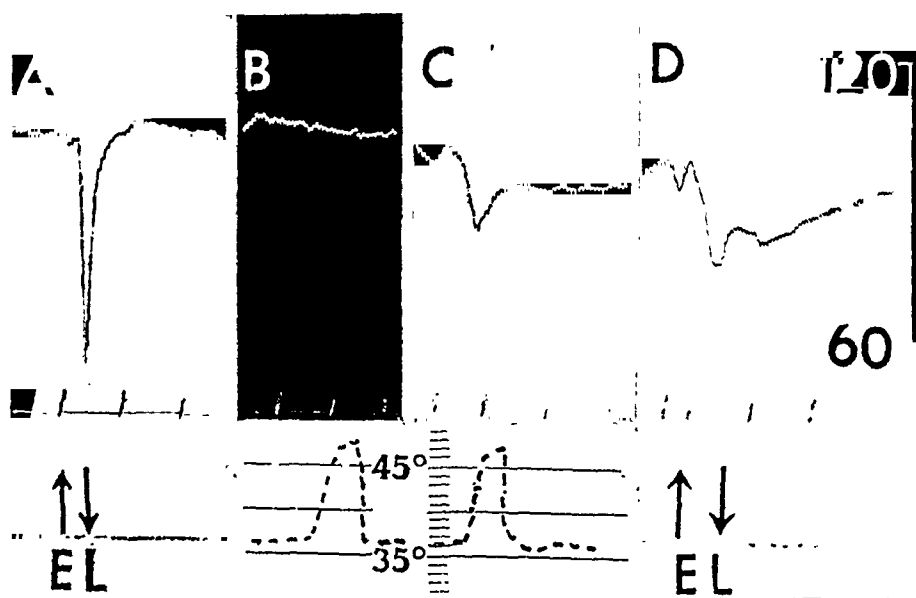


Fig. 19. Rabbit, Dial. Blood pressure (mm Hg). Dotted line: thermode temperature. Vagus nerve in the neck. A, electrical and B, heat stimulation of the peripheral end of the severed nerve. C, heat and D, electrical stimulation of the central end of the same nerve. Time in 30 sec.

affecting the surrounding tissues by thermal or electrical stimulation. Regular controls were made to ensure that this did not occur.

Results. If the thermode was placed on the peripheral end of the severed vagus nerve in the neck and then heated no change occurred in the blood pressure. Neither did cooling produce any effect on the blood pressure. However, electrical stimulation produced the classical response, a marked decrease in blood pressure. If the blood pressure was recorded with an elastic manometer and the kymograph speeded up so that the frequency of the heart beats could be clearly determined, it was found that neither heating nor cooling of the peripheral end of the vagus ever succeeded in influencing the heart frequency. Electrical stimulation had the characteristic inhibitory effect. The decerebrated cats and the rabbits anaesthetized with Dial reacted similarly (fig. 19).

The afferent fibres of the vagus nerve in the neck were studied in rabbits. On these animals the vagus, for some distance, runs separately from the cervical sympathetic trunk and the depressor nerve. The effects varied for different animals but were similar for each experiment. Sometimes a pure pressor effect was obtained by heating but more often, a pure depressor effect. In some animals the effects were mixed (see fig. 19). Different effects were obtained with electrical stimulation depending on the frequency of the stimuli, and variations were also encountered with different animals (see McDOWAL, 1935). Only in one case a small depressor effect was obtained with cold.

If the thermode was placed on the vagus in the thorax distal to the branching of the recurrent nerve and the branch to the heart, but proximal to the branches to the lungs a depressor effect was always obtained on heating (decerebrated cat, fig 20), but on cooling no vasomotor reflex was evoked. Neither heating nor cooling elicited any change in the motility of the stomach. Strong electrical stimulation at this level elicited the same vasomotor reaction as heat, but in addition a marked contraction of the stomach in agreement with BROWN and McSWINEY (1932). With thermal stimulation of the vagus nerve immediately above the diaphragm, a vasomotor reaction was never obtained nor was there any effect on the motility of the stomach. Electrical stimulation at this level using the same strength and frequency (75/sec) as before, elicited no vasomotor reaction, but there was a contraction of the stomach (fig. 20).

Great splanchnic nerve.

Fig. 21 shows a typical example of the experiments made on the great splanchnic nerve. In A and B the thermode is placed on the intact nerve. A gives the effect of heating, a small pressor effect. B shows a large pressor effect elicited by a strong electrical stimulus with a frequency of 75/sec. In C and D the nerve is ligated and cut, the thermode is placed on the peripheral stump. C shows that no effect on the blood pressure was elicited by heat, while D shows that an electrical stimulus of the same

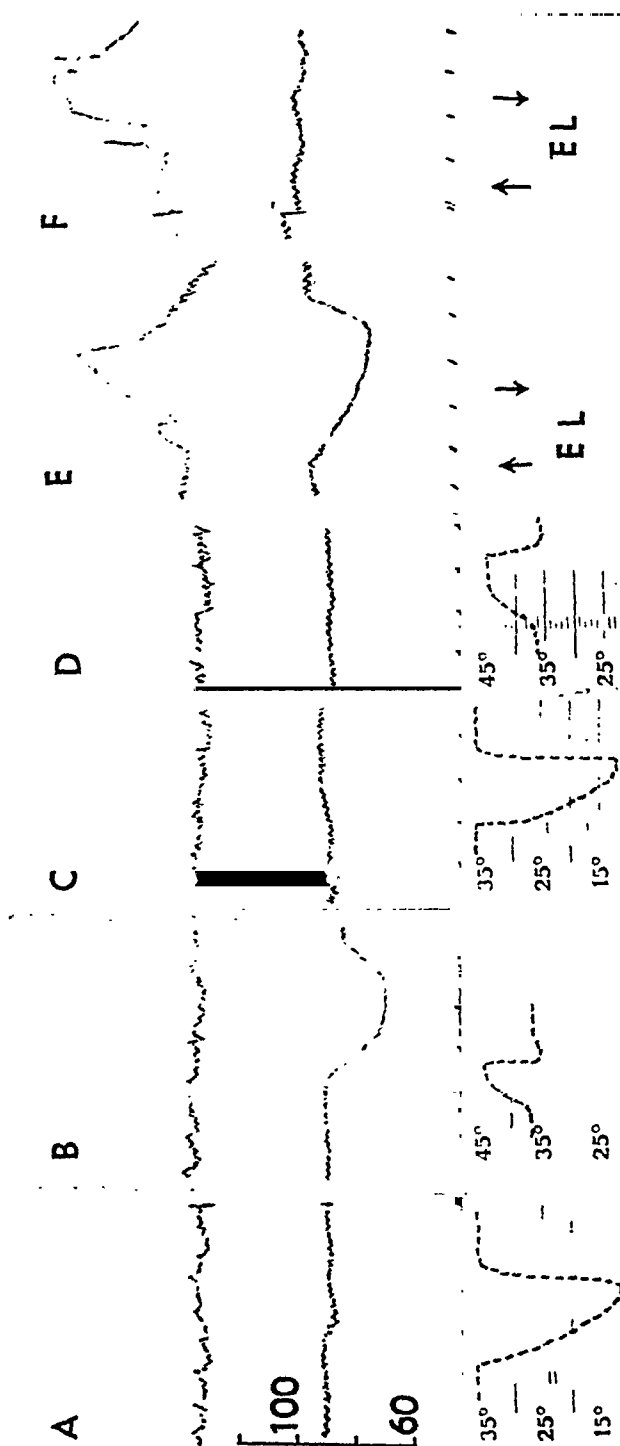


Fig. 20. Cat, decerebrate. Upper tracing: tone of the stomach, lower tracing: blood pressure. Vagus nerve in the thorax. Artificial respiration. A, and B, thermal stimulation of the intact vagus nerve between the cardiac and the pulmonary branches. C, and D, thermal stimulation just above the diaphragm. E, electrical stimulation at the same level as in A and B. F, electrical stimulation at the same level as in C and D. See text. Time in 10 sec.

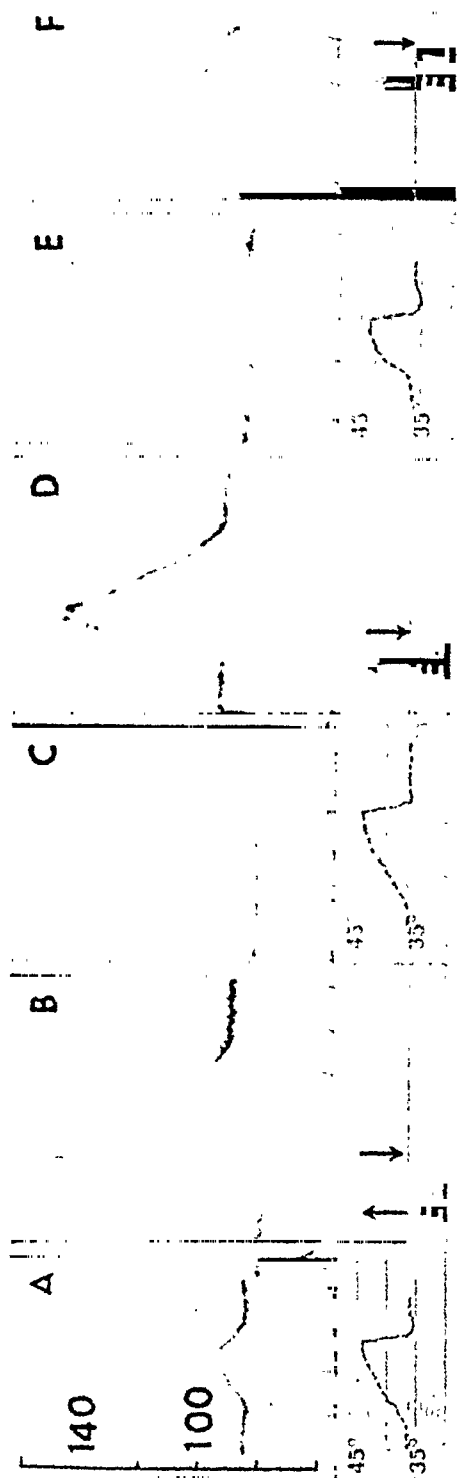


Fig. 21. Cat, decerebrate. Blood pressure (mm Hg). Dotted lines: thermode temperature. A, thermal and B, electrical stimulation of the intact great splanchnic nerve. C, thermal and D, electrical stimulation of the peripheral end of the covered splanchnic. E, thermal and F, electrical stimulation of the central end of the same nerve. See text. Time in 10 sec.

strength as in B, elicits very nearly the same pressor effect as on the intact nerve. In E and F the thermode has been moved to the central stump of the same nerve. Here, heat (D) and electrical stimulation (F) of the same frequency as in B and D, elicit equivalent pressor effects. In some of these experiments the motility of the stomach was also registered. Strong electrical stimulation with a frequency of 75/sec., caused a relaxation of the stomach, but in no case did thermal stimulation elicit any reaction of the stomach.

Cervical sympathetic trunk.

According to BISHOP and HEINBECKER (1933) the cervical sympathetic trunk in the rabbit consists mainly of small myelinated pre-ganglionic fibres of the B class. In addition there are post-ganglionic fibres from the upper cervical ganglion to the heart. Afferent fibres have not been demonstrated.

Thermal and electrical stimulation of the cervical sympathetic trunk has been studied on 12 rabbits, with respect to the responses from the nictitating membrane and the reactions of the ear vessels. *Technique.* The cervical sympathetic trunk was freed for a sufficiently long section so that it could be placed in the thermode without stretching. In most cases the nerve was ligated and cut about 2 cm. distal to the thermode. There was, however, no difference in the effects obtained whether the nerve was severed or not. Controls were made after each experiment to show that none of the effects elicited were due to the spreading of the thermal or electrical stimulus to the surrounding tissues.

The reactions of the blood vessels in the ear were recorded by means of a pletysmograph. The ear root was shaved and a double-walled glass pletysmograph was placed over the ear and sealed with vaseline. Changes in volume were registered with a piston recorder.

In order to record the contractions of the nictitating membrane the head of the rabbit was fixed so that the membrane would stretch vertically upwards. The edge of the membrane was attached to the short end of a writing lever with a thread. In

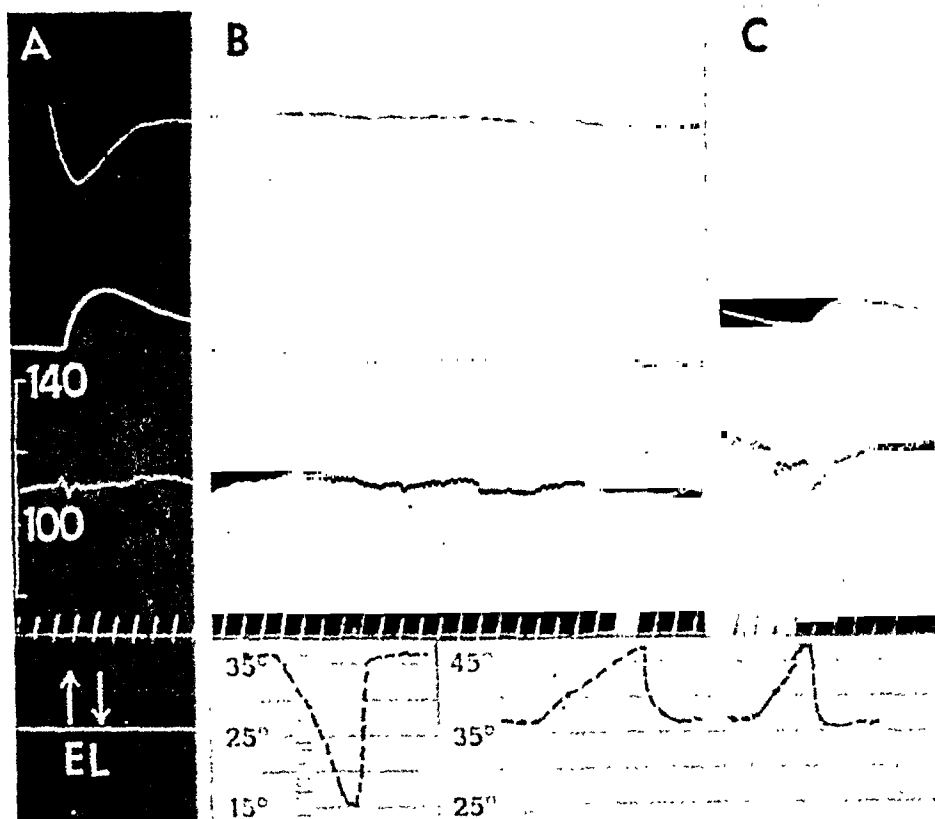


Fig. 22. Rabbit, Dial. Upper tracing: volume of the ear (increase: upward deflection). Middle tracing: contraction of the nictitating membrane. Lower tracing: blood pressure. Dotted line: thermode temperature. Time in 10 sec. A, electrical and B thermal stimulation of the intact cervical sympathetic trunc. C, thermal stimulation of the saphenous nerve eliciting a reflex contraction of the nictitating membrane.

most cases, measurements of changes in ear volume, contractions of the nictitating membrane and the blood pressure were registered simultaneously.

Results. Neither local heating nor cooling had any effect on the blood pressure, the vessels of the ear or the nictitating membrane. Strong electrical stimulation at a frequency of 96/sec. always evoked vaso-constriction of the vessels of the ear and contraction of the nictitating membrane (fig. 22). The effects on the blood pressure of this stimulus have been small and irregular, even when stimulating the peripheral end of the severed nerve.

The reaction of the pupils was also observed in these experiments but the alterations in the diameter were not measured or registered. Electrical stimulation which evoked reactions in the nictitating membrane and the vessels of the ear, elicited a dilatation of the pupil on the same side. In none of these experiments did heat or cold cause any alteration of the pupil.

Perivascular efferent nerves to the small intestine.

In the above experiments, principally pre-ganglionic fibres have been investigated. For a study of the post-ganglionic fibres a smooth muscle nerve preparation described by FINKLEMAN (1930) was chosen.

Technique. The preparation consists of a strip of small intestine of rabbit with adherent nerves. The rabbit was killed by an intravenous injection of air. The abdomen was opened and a piece of small intestine about 5 cm. long was removed together with the mesenterial artery supplying it. The nerves to the intestine follow the arteries very closely. The artery with its peri-arterial nerves was freed as much as possible from the surrounding tissues. In order to record the movements of the intestinal section, the lower end was fastened to the bottom of a bath (50 ml) and the upper end to the short end of a writing lever by means of a vertical thread. The bath was filled with Tyrode solution aerated with carbogen (5 % CO_2 in oxygen, to maintain a constant pH). The bath was kept at a constant temperature of 37° by means of a thermo-regulator. The nerve (and artery) was placed in the thermode and onto stimulation electrodes, the thermode being placed between the electrodes and the intestinal stump. The stimulation electrodes were connected to a thyratron stimulator. In all, 13 smooth muscle nerve preparations were studied.

Results. In agreement with McSWINEY and ROBSON (1931) and BROWN and McSWINEY (1932), the electrical stimulus elicited only an inhibition of intestinal movement and relaxation, independent of the frequency used. In no case could any increase in

EL 47° 36° 10° 36° EL.
 ↓ ↑ W ↓ C ↓ C ↓ ↓ W ↓ ↓ ↑

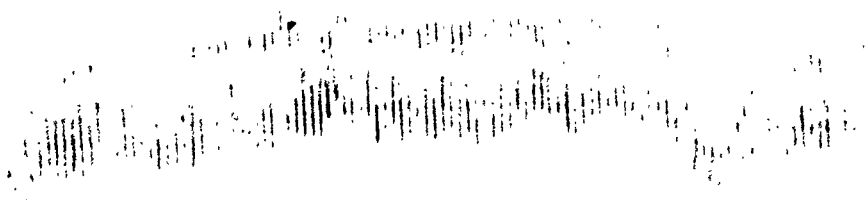


Fig. 23. Rabbit. Jejunum. Thermal and electrical stimulation of the periarterial nerves. EL=electrical stimulation (freq. 75 per sec.). At W, warming starts, at C, cooling starts. See text.

intestinal movement be obtained. Inhibition and relaxation due to electrical stimulation of the peripheral nerves to the intestine depends, as McSWINEY and ROBSON have shown, on the excitation of the post-ganglionic sympathetic nerve fibres.

With thermal stimulation no effect on intestinal movement was ever obtained, neither by heating to 47° nor by cooling to 10° (fig. 23).

In a few cases the nerve (and artery) was excited electrically during thermal stimulation. Fig. 24 shows such an experiment. A shows that heating to 47° had no effect, but, keeping the temperature at this level, electrical stimulation resulted in a marked inhibition and relaxation. B shows the effect of the electrical stimulus at a thermode temperature of 25°. The effect is less than in A. In C the nerve is cooled down to 15°, thereafter the electrical stimulus is applied. The effect is now further decreased. In D, the thermal temperature is 10°. Here the effect of the electrical stimulus is practically annulled. E gives the effect at a temperature of 36°. Now the effect of electrical stimulation is once again increased.

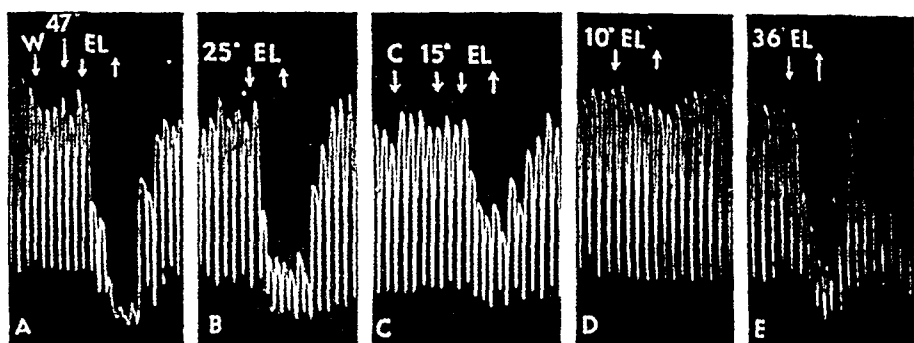


Fig. 24. Rabbit. Jejunum. Thermal and electrical stimulation of periarterial nerves. The thermode is placed between the stimulating electrode and the intestine. EL=electrical stimulation. At W, warming starts and at C, cooling starts. See text.

Discussion.

The experiments on the vagus nerve support the opinion (p. 48) that this nerve, in the lower thoracic region, contains few if any thin afferent fibres. Neither thermal nor electrical stimulation of the vagus elicited any vasomotor reflexes.

The efferent fibres in the upper region of the vagus nerve which conduct vasomotor reflexes, have already been shown to be of about the same size as the efferents to the heart and the stomach (cf. above p. 48). The experiments demonstrate that the same electrical stimulus which elicited effects from both heart and stomach, evoked, via the afferents, vasomotor reflexes equivalent to those obtained by heating. But heating had no effect on the efferent fibres of this nerve.

The results obtained from the great splanchnic nerve shows the same. Stimulation of the central end of the cut nerve by electrical or thermal means elicited equal effects, but on stimulation of the efferent, peripheral end, the two forms of stimulation differed in that the thermal one was quite ineffective.

The thermal inexcitability of the vegetative motor fibres is further demonstrated by the experiments performed on the cervical sympathetic trunk, and on the postganglionic fibres of the smooth muscle-nerve preparation. Analysis of the latter pre-

paration also showed that local heating of these fibres did not block them. It will be remembered that BREMER and TITECA (1946), using the frog sciatic, found that heating of the *whole* nerve in a paraffin bath abolished the large fibre component of the electroneurogram.

The main conclusion to be drawn from these results is that there is a marked and important difference in sensitivity to thermal stimulation between, on the one hand, thin myelinated and unmyelinated *afferent* fibres and, on the other hand, the thin myelinated and unmyelinated autonomic *efferent* fibres, despite their corresponding histological properties.

The mechanism behind this difference will be further illustrated by experiments on the temperature potentials in these nerves.

Temperature potentials

When BERNHARD and GRANIT (1946) had noted that not only the locally cooled but that also the similarly heated region of a nerve became electronegative relative to the rest of the nerve they suggested that these thermopotentials served as 'generator potentials' for the discharge which they had recorded in response to heating and cooling. Now, however, we have seen that cooling and heating selects fibres of different size and consequently it has become imperative to find out whether also the heat and cold potentials possibly might be generated differentially in the fibres that responded with impulse activity to heat and cold respectively.

In order to investigate this problem it was decided to try to obtain nerves with fibres of as homogenous size as possible. It has previously been stated (p. 48) that the vagus nerve, in the lower part of the thoracic cavity immediately above the diaphragm, is almost entirely composed of unmyelinated fibres; also that the phrenic nerve (p. 36) and the recurrent nerve (p. 37) contain but a very small number of the thinnest fibres whereas the cervical sympathetic trunk (p. 54) principally consists of the thinnest myelinated fibres, which belong to the B class, in addition to a few unmyelinated fibres. The cervical sympathetic trunk in the cat contains also a small number of δ fibres (BISHOP and HEINBECKER, 1930, GRUNDFEST, 1939). The vagus nerve in the neck consists both of thick and thin fibres, the latter in the majority. Here then are nerves which would seem suitable for an analysis of the temperature potentials as functions of fibre size. These potentials have been measured, partly on nerves in situ and partly on nerves removed and kept in Tyrode solution.

Technique. The temperature potentials of nerves in situ were studied in about 20 cats. The animals were decapitated under ether, the spinal cord being cut between C_2 and C_3 , then given artificial respiration with a Starling pump and left to expire

the narcotic during 2 hours. The vagus nerve in the neck and the cervical sympathetic trunk were carefully dissected from each other, avoiding any stretching or pinching. The phrenic nerve and the distal part of the vagus nerve were layed free in the thoracic cavity opened by the removal of the fourth to twelfth ribs inclusively, usually on the right side. In order to avoid stretching when placing the vagus nerve in the thermode it had to be cut at the level of the cardia. The animal was placed in a box of constant temperature (36°) and saturated humidity.

Ag-AgCl electrodes with long cotton wool wicks moistened with Ringer solution were used. One electrode was taken to the nerve in the middle of the thermode, the other one to a thermally indifferent point on the animal. The electrodes were connected to a recording instrument consisting of two push-pull coupled electrometer valves operating a moving coil mirror galvanometer with a period of 8 seconds. The mirror reflected a light beam onto a scale graded in $1/10$ mV. With this system a steady base line was obtained.

In these experiments it proved important to have the thermode electrically well insulated. This was attained by coating the silver thermode several times with a special enamel and drying it in an oven at a temperature of 300° after each coating. In this way a thin and very hard film with good electrical insulation was obtained.

When the nerves were analyzed in the bath, they were removed from a decapitated cat a couple of hours after the narcosis. The vagus nerve was dissected out from just below the nodose ganglion to a point at about the level of the cardia with the recurrent nerve attached; practically the whole phrenic nerve could be isolated. The nerves were placed in a Tyrode bath aerated with carbogen (5 % CO_2 in oxygen) so as to have a pH of 7.0. The temperature of the bath was kept constant at 37° . The same temperature and saturated humidity surrounded the bath in the box where it was placed. During the experiment the thermode was placed just above the surface of the solution, one electrode being on the nerve in the thermode, the other one dipping down into the bath.

Results. All measurements were begun at 37°. From this level the thermode temperature was either raised or lowered and the resulting potential read off. In each experiment a series of measurements were made at different temperatures and the average value of the thermo-potential for each temperature was calculated. The results of the different experiments were then combined in the diagrams shown in figs. 25—27. During these experiments, it was not attempted to keep the temperature gradient absolutely constant; too large variations were, however, avoided by maintaining the containers for hot and cold water at approximately constant temperature and circulating the thermodes at maximal current velocity.

The cervical sympathetic trunk (see fig. 25) reacted with an electro-negativity of between 1 and 1.5 mV at the heated region relative to the thermoindifferent point. In two cases this "heat potential" exceeded 3 mV. In both these experiments the "cold potential" also exceeded by about 1 mV its normal very low value of between 0 and 0.3 mV. On return to the initial temperature electrical equilibrium was generally restored almost completely, except in those cases where the higher "heat potentials" had been observed. There the restorative process was incomplete during the nearest minute. The cervical sympathetic was studied in six animals, on all in situ.

The vagus nerve immediately above the diaphragm was studied on 14 nerves in situ, on 6 nerves in the Tyrode bath. In one case a nerve in situ evoked a cold potential greater than 1.5 mV. And in 2 cases cold potentials of 1.0 mV were obtained. In one of these a positive heat potential appeared. In the remaining 11 cases heat elicited a definite negative potential (see fig. 26), while cold produced none or very small potentials. On return to normal temperature the galvanometer usually swung back almost to zero or fully so, but in a few cases the restoration of equilibrium was very incomplete. The nerves in Tyrode solution generally reacted similarly except that the potentials were lower and the values from different experiments less scattered. Thus this part of the vagus nerve can obviously generate marked heat

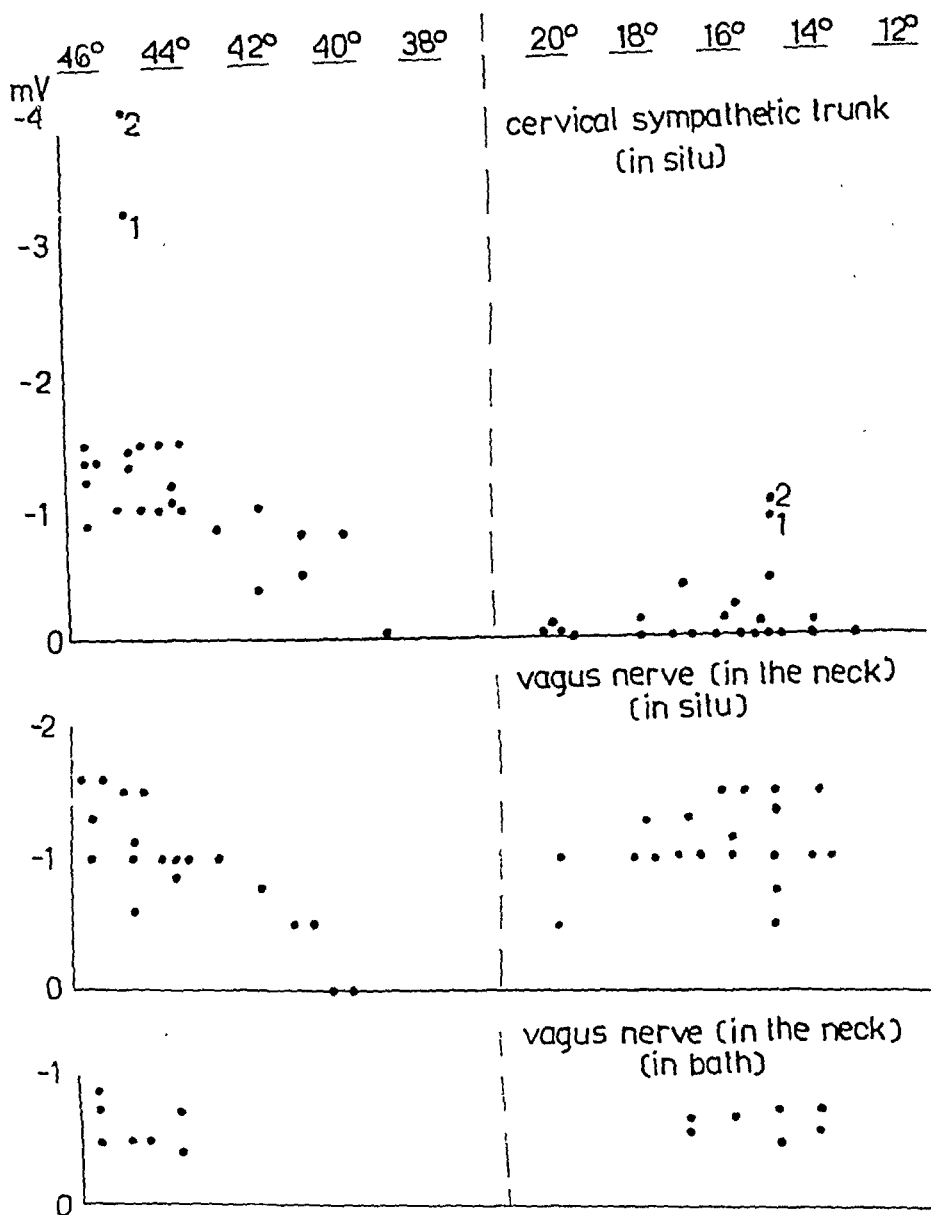


Fig. 25. Temperature potentials. Each dot represents the average of the values obtained from the same nerve. Dots of the same number traced from the same nerve. See text.

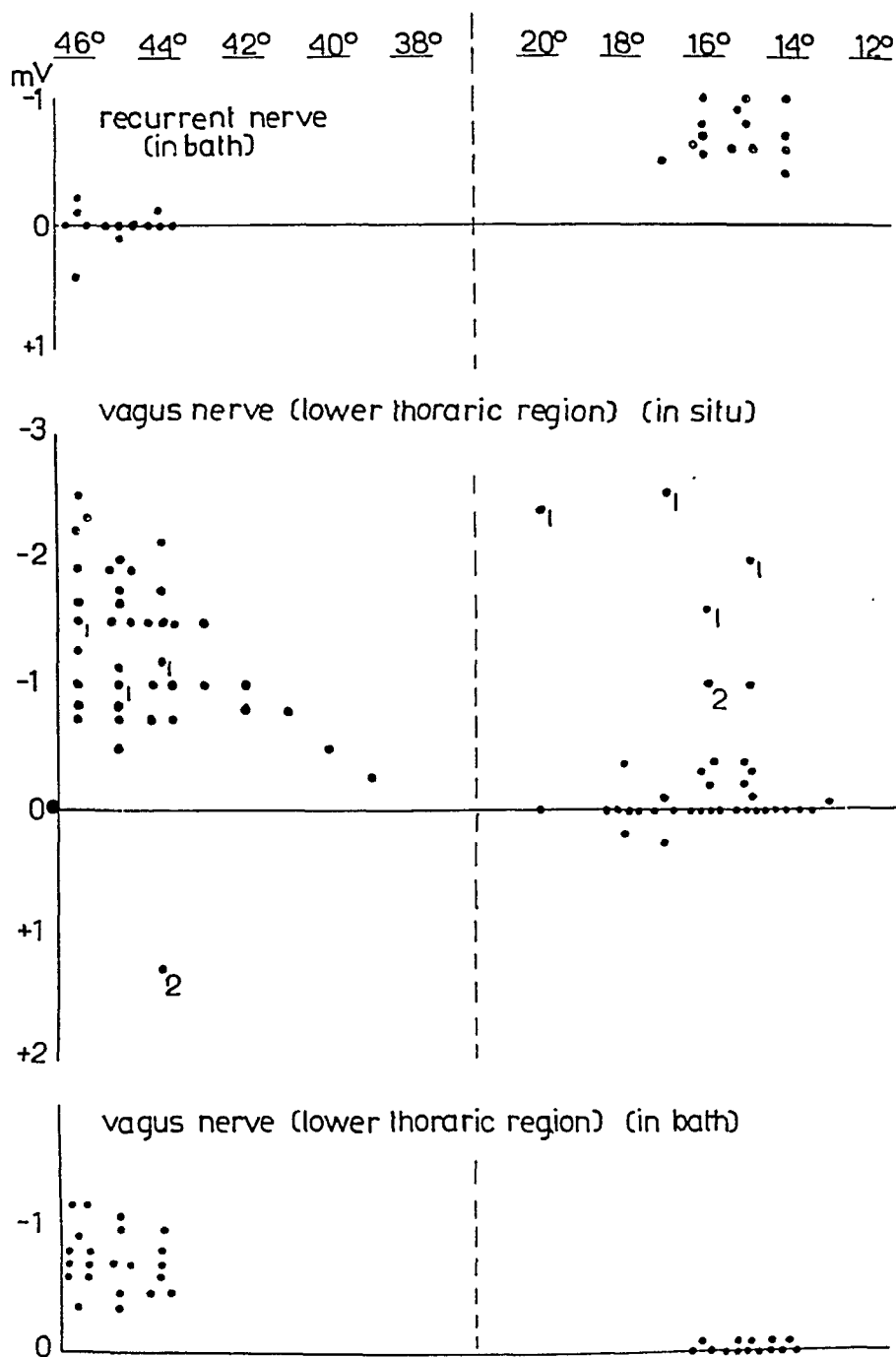


Fig. 26. Temperature potentials (as in fig. 25). See text.

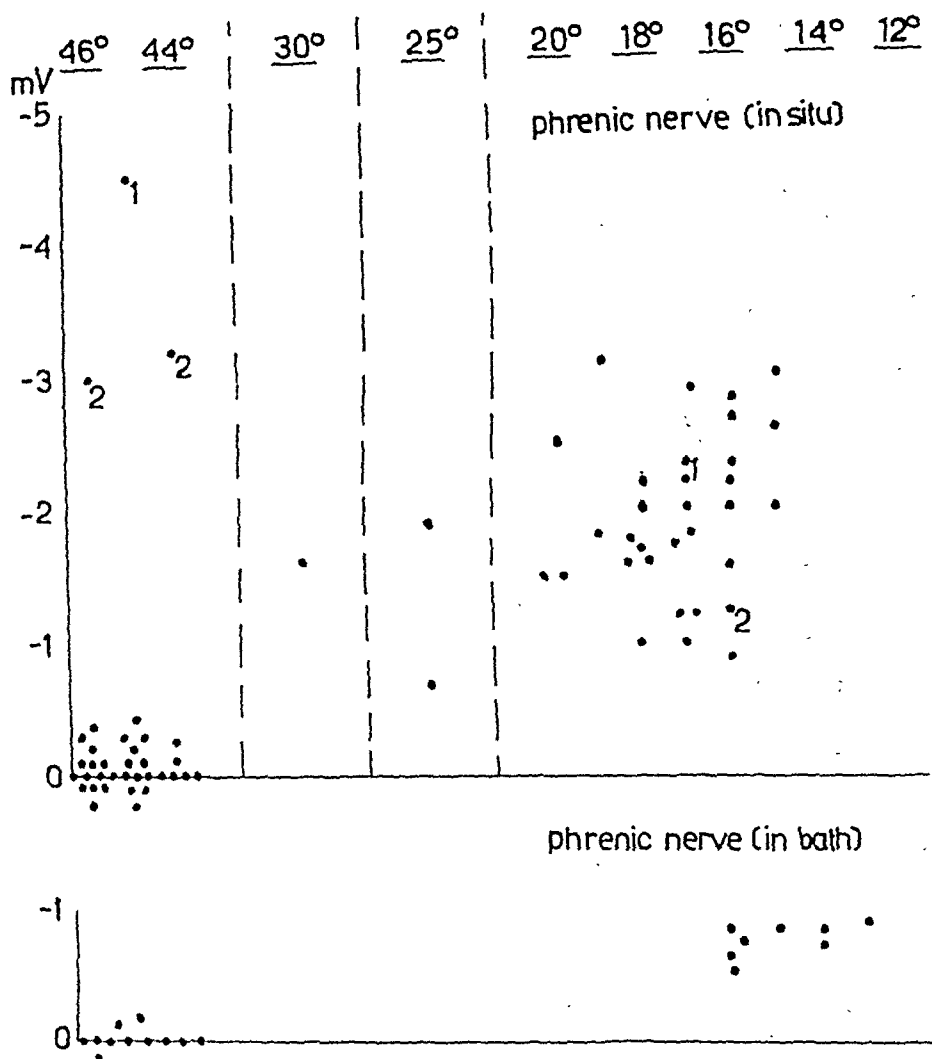


Fig. 27. Temperature potentials (as in fig. 25). See text.

potentials, while cooling elicits no response or very small disturbances of potential.

The *vagus nerve in the neck* was investigated in situ on 6 animals and removed from 4 animals for analysis in the bath (see fig. 25). In this nerve, both heat and cold set up definite negative potentials of about the same size. As in the other cases the nerves in situ responded with larger potentials than those in the bath, otherwise the reactions were similar. After the temperature had

returned to its initial value, the restoration of potential equilibrium was practically complete.

Twelve *phrenic nerves* (see fig. 27) were analyzed in situ and four nerves in the bath. Amongst the nerves studied in situ two responded with quite large heat potentials while in all the other cases the values were negligibly small and fell between 0.4 mV neg. and 0.2 mV pos. In all cases cooling evoked a marked negative potential of about 1—3 mV. On return to normal temperature potential equilibrium was restored in most cases to within 1/10 mV. It was sometimes observed, that just as the temperature began to rise from its cold minimum, the negative potentials increased further, and then suddenly returned and even swung over to a small positive value.

The same small heat and large cold potentials were obtained in the experiments on the phrenic nerve in the bath. The values were smaller but less scattered. There was a marked cold potential of about 1 mV but none or at least a much smaller heat potential. The *recurrent nerve* was removed from 4 animals (5 nerves) and studied in the bath. This nerve behaved like the phrenic nerve. The heat potentials varied between 0.2 mV neg. and 0.2 mV pos. while the cold potentials varied between 0.4 mV neg. and 1.0 mV neg. (see fig. 26).

After every experiment, controls were made in an exactly identical manner except that the nerve was exchanged for a cotton thread, moistened with Ringer and placed in contact with the animal as if it had been a nerve. In 3 cases a small positive potential of about 0.1 mV was observed on heating, and a negative potential of up to 0.3 mV on cooling. The values obtained for the temperature potentials were corrected with the aid of the controls.

Discussion.

The idea that the temperature potentials set up by heating or cooling the nerve generate the discharge to heat or cold in respectively thin and thick fibres necessitated the further assumption that different fibres also generate the heat and cold potentials. So far this theory has stood the test of experiment. The capacity

to generate thermopotentials in response to heat or cold has been found to differ according to whether the nerves tested have been dominated by thin myelinated and unmyelinated fibres or thick myelinated fibres. The thick fibres have not been capable of generating significant thermopotentials to heat but have readily responded to cold with a large negative deflexion at the cooled region. It was shown above that these are the very fibres that under such circumstances also discharge impulses. The thin fibres which discharge in response to heating proved to give large thermopotentials to this particular stimulus and were relatively indifferent to cooling.

It is true that these experiments on thermopotentials have been restricted to vegetative efferents. However, the discharge in the somatic afferents was shown above to be similarly differentiated with respect to heat and cold and somatic afferents have been found by BERNHARD and GRANIT (1946) to generate both heat and cold potentials. It is therefore probable that the two kinds of somatic thermopotentials also are generated in different fibres, the heat potentials largely in unmyelinated and myelinated thin afferents, the cold potentials primarily in large myelinated fibres.

In the phrenic nerve there is a sufficient number of thin myelinated fibres to give a small but clearly detectable blood pressure rise. It has, however, not been possible to demonstrate the expected heat potential. The reason for this might be the limited number of heat sensitive fibres. Their response to heat has probably not been able to reach the instrumental threshold.

Similarly in the cervical sympathetic trunk there is a small number of δ fibres. If these have been capable of setting up a cold potential it is, for the same reason, probable that it has remained subthreshold with regard to the absolute level of sensitivity determined by the preparation and the recording technique.

On the galvanometer scale 0.1 mV has corresponded to 1.5 mm. This should be ample but since the experimenter has had to read off both temperature and thermopotentials the accuracy of the measurements has suffered somewhat from this splitting of his attention between two rival tasks. For a qualitative survey the

measurements should, however, be satisfactory. Other factors, beyond experimental control, have probably caused greater errors, particularly in the experiments on intrathoracic nerves *in situ* where odd values fell quite outside the normal limits of variation. Although the absolute values of the potentials of isolated nerves in the Tyrode bath were smaller they were more regular.

This circumstance suggests that the amount of surrounding tissue is of importance. The isolated nerves could be very much better cleaned and, in general, the experimental conditions with this technique were more stable and homogeneous. The nerves *in situ* could never approach the same degree of uniformity. But even if the nerve itself were a fairly homogeneous structure with regard to its fibre composition it is by no means certain that every response to changes of temperature is due exclusively to the conducting structures or the so-called nerve membranes. There are also the sheaths of connective tissue and the vasa nervorum to consider all of which contain membranes as well as electrolytes which there are no *a priori* reasons for regarding as thermoelectrically indifferent. Thermopotentials are common enough. It is, for instance, merely necessary to shorten the cotton wool wick of the cooled or warmed electrode so that the temperature change is conducted to the silver-silverchloride electrode itself in order to record a large artefact electrode potential in response to heat and cold. With sufficiently long cotton wool wicks this source of error can be avoided. But it shows that caution should be exercised when ascribing thermopotentials to definite sources. The non-nervous part of the tissue may have been responsible for the small and irregular cold potentials in nerves containing thin fibres and for the similar heat potentials in nerves dominated by thick fibres.

The differences here found between small and large fibres with respect to their capacity to generate thermopotentials are probably due to specific membrane properties or to differences in electrolytic composition. Myelination as such cannot explain it because the myelinated cervical sympathetic and the unmyelinated distal portion of the vagus behave in a similar fashion.

The failure of GRÜTZNER (1881) and VERZÁR (1911) to demonstrate that a heated portion of a nerve becomes electronegative relative to a part at normal temperature can probably be explained by their having used frog nerves. Nor did they attempt to keep the nerves in anything like its normal environment. Systematic analyses of the effect of the environment on thermopotentials have been started in this laboratory.

The thermopotentials need not necessarily succeed in setting up a discharge of impulses. The latter mechanism may be disturbed or the nerve may accommodate very rapidly to the potential change and for this reason be unable to discharge. ERLANGER and BLAIR (1938) have shown on frogs and SKOGLUND (1942) on cats that large sensory fibres accommodate far less than motor fibres of the same size. That similar differences exist between the thinnest sensory and the autonomic motor fibres is not improbable since C. v. EULER (1947) has noted (on cats) that the unmyelinated motor fibres in the vagus are very insensitive to stimulation with a slowly rising current. It was found impossible to elicit a repetitive discharge from these fibres although the strength of the current was increased to more than 4 times the rheobase. On the other hand, the thin sensory fibres of somatic nerves were quite sensitive to stimulation with a slowly rising current. This indicates that there is more accommodation in the vegetative efferents than in sensory fibres of comparable size. The fact that thermopotentials in vegetative efferents do not set up impulse activity could be satisfactorily explained by the high accommodative properties in these nerves.

General Comments

It has been shown in this paper that local heating, e.g. of the sciatic nerve, represents a simple method for eliciting a small fibre reflex increase of blood pressure without excitation of the large fibres. This observation can be put to practical use in investigations of the vasomotor centres, as for instance with regard to the pharmacodynamic properties of different agents. The method was used above (p. 21) in investigating the effect of ergotamin in small doses on the thermally elicited pressor reflex.

The fact that only the afferents of a visceral nerve and not its efferents discharge in response to heating supplies a new method for the analysis of reflexes conducted through the intact nerves of various organs as long as the temperature used does not block the efferents of the same nerve. It has, however, been shown (p. 57) that a temperature of 47° does not block post-ganglionic fibres to the small intestine, and it can be assumed that other autonomic efferents behave similarly.

Thus this selective method of generating thermopotentials and impulses in the thinnest fibres of a nerve offers new opportunities for the study of these fibres. Their properties and functional projections represent an important field for investigation, especially with respect to problems connected with the autonomic nervous system.

Summary.

The responses evoked from mammalian nerves by local changes in temperature have been studied by means of the thermode technique.

1. Local heating of the sciatic nerve of the decerebrated cat elicited a complex nociceptive reflex pattern involving skeletal and vegetative motor effects. The investigation has particularly dealt with the vasomotor and, to some extent, the respiratory reflexes set up in this manner. Curare, in doses paralysing the respiratory movements, did not affect the vasomotor reflexes, nor did ergotamin, in small doses, affect the pressor reflexes.
2. The calibre spectra of different somatic nerves were correlated with the vasomotor (sometimes also the respiratory) reflexes, elicited by local heating and cooling. It was found that local heating of a nerve excited the thinnest afferents of the δ group and the C class. Cooling did not excite these fibres but stimulated the thick myelinated afferents.
3. Cooling was shown to stimulate large myelinated motor fibres, while heating did not excite these fibres.
4. Action currents obtained in response to stimulation by heating and cooling of the thin branches of sensory nerves were studied. It was found that heating did not to a measurable degree excite afferents thicker than those of the δ group, and that cooling stimulated thick myelinated afferents.
5. It was shown that neither heating nor cooling of the autonomic efferent fibres elicited any effects from the innervated organs. Somatic *afferent* and vegetative *efferent* fibres, although of practically the same size, therefore differ with regard to their capacity to discharge in response to local heating.
6. The local potential changes caused by local changes of thermode temperature were measured electrometrically on different nerves, as homogenous as possible with regard to fibre composi-

tion. These investigations showed that in such nerves local heating of thick myelinated fibres was ineffective or merely gave rise to very small potential changes whereas local cooling regularly caused marked local negative potentials (relative to a thermo-indifferent point). Unmyelinated and thin myelinated fibres, on the other hand, regularly gave rise to marked negative thermopotentials in response to heating but remained practically isoelectric when cooled.

7. These results are held to substantiate the view that the discharge of impulses in response to heating in thin fibres, to cooling in thick fibres is caused by their respective "heat" and "cold" potentials serving as "generator potentials".

8. In view of the presence of generator, "heat" potentials in both the thin sensory and the thin motor fibres, the difference between these fibres with regard to their capacity to discharge in response to heating is considered to be a question of differences of accommodation.

9. The applicability of thermostimulation as a method of selecting small fibres and as an index of small fibre activity is being discussed.

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FROM THE MEDICAL CLINIC AND ITS ROCKEFELLER LABORATORY
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STUDIES ON THE TRANSPORTA- TION AND METABOLISM OF IRON IN THE BODY

WITH SPECIAL REFERENCE TO THE IRON-
BINDING COMPONENT IN HUMAN PLASMA

BY

CARL-BERTIL LAURELL

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Preface.

The present investigation was carried out in the Medical Clinic in Lund and its Rockefeller Laboratory during the period of 1945—1947. To the former chief of this clinic, the late Professor Sven Ingvar I owe a debt of gratitude for his interest in my work and his kindness in affording me some most excellent working conditions.

This subject was taken up at suggestion of Docent Carl Gottfrid Holmberg. His comprehensive knowledge of — and deep insight in — the present problems have been of invaluable help to me, and in the progress of my studies his never-failing interest and lucid suggestions have been of great benefit to me.

I further wish to acknowledge my indebtedness to all the chief physicians — in particular Docent Knut Liedholm —, assistant physicians, nurses and comrades in the hospital who have supplied me with clinical material.

To Dr. Georgs Punnenovs and Mrs. Gulli Lundberg I am deeply indebted for their painstaking and efficient assistance.

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Finally, I thank Dr. Hans Andersen, Copenhagen, for his interest and care in translating my work into English.

Lund, August 1947.

Carl-Bertil Laurell.

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INTRODUCTION.

Literary Survey of Recent Data on the Intermediary Iron Metabolism with special Reference to Serum as Vehicle for the Transport of Iron.

During the last decade our knowledge of the iron metabolism has been extended considerably through studies on variations of serum iron under pathological and physiological conditions and through the detection of ferritin — a protein capable of binding up to 23 % iron and thus serving as a storage-iron fraction. After radioactive iron has commenced to be employed as tracer, new observations have thrown additional light on the way in which the organism regulates, among other things, the absorption and excretion of iron as well as the hemoglobin metabolism.

In the present work an account is given of the quantitative variations of the iron-binding protein component in serum in normal subjects and in patients suffering from various diseases. The significance of this protein fraction to the transport of iron in the organism is elucidated with experimental and clinical observations, partly in connection with previously established facts concerning the iron metabolism.

Distribution of Iron in the Organism.

»The total iron in a 70-kg. man averages about 4.3 g. Of this iron, some 55 per cent is contained in the circulating hemoglobin, and about 10 per cent is a constituent of muscle hemoglobin and the hemecatalysts. The remainder, 30—35 per cent of the body iron, may be designated as the storage-iron fraction. About two-

thirds of this storage iron, or 0.8 g., is located in the liver, spleen and red bone marrow» (Granick 1946). The transport iron fraction (serum iron) amounts only to about 1 % of the body iron.

The iron metabolism has to be considered on the background of the fact that iron which once is incorporated in the body is retained, and that the physiologically active iron is utilized in a narrow cyclic process (hemoglobin — serum iron — hemoglobin) rather independent of absorption, excretion and storage.

Excretion of Iron.

The organism appears practically incapable of active iron excretion — as has been established, among other ways, through experiments with parenteral administration of radioactive iron (McCance & Widdowson 1937, 1938 and 1943, Hahn, Bale, Hettig, Kamen & Whipple, 1939, Brownlee, Bainbridge & Thorp, 1942, Greenberg, Copp & Cuthbertson, 1943, Copp & Greenberg, 1946). Thus no active elimination of iron takes place through the kidneys. On intravenous injection of large doses of iron there is a percentally insignificant excretion of the iron with the urine in the first hours after the injection, and then the renal elimination falls off towards 0. Normally the diurnal excretion of iron with the urine is less than 1 mg. [for literature, see Heath (1942), Dahl (1944)], although within the same period about 1 g. iron passes through the kidneys in the form of serum iron.

It has also been shown that no active excretion of iron takes place in the intestinal canal on parenteral administration of iron. Further, under physiological conditions the elimination of iron by way of the bile passages is minimal (*e.g.* Hahn *et al.* 1939, Greenberg *et al.* 1943).

The insignificant loss of iron by way of the intestinal canal is relatively constant and may be attributable to detachment of epithelial cells.

As the organism thus is practically unable to eliminate the iron once incorporated, it has to be capable of regulating the iron absorption — lest it be flooded with iron.

Absorption of Iron.

Hahn, Bale, Lawrence & Whipple (1938—1939) have been able to demonstrate that quite insignificant amounts of iron are absorbed under physiological conditions.

In simple anemia from iron deficiency the absorption of iron given by mouth is many times greater than in a normal subject. The greater the iron ingestion, the lesser is its percental absorption. If two doses of iron are given at an interval of a couple of hours, a part of the first dose is absorbed, whereas the second dose passes through the digestive tract practically without being utilized at all (*e.g.* Hahn, Bale, Ross, Balfour & Whipple 1943).

These observations are usually interpreted to the effect that the organism is able by way of the intestinal mucosa actively to regulate its iron absorption after its need. When the iron requirement is increased (*e.g.*, after a hemorrhage) the relative blockade of the iron absorption normally exercised by the intestinal mucosa is abolished (*e.g.* Balfour, Hahn, Bale, Pommerenke & Whipple 1942, Chapin & Ross 1942).

Storage-iron Fraction.

In connection with his studies on »ferritin, its properties and significance to the iron metabolism», Granick (1946) has given an excellent review of our present knowledge of the storage-iron fractions.

The storage-iron fractions may be divided into two parts: colloidal iron compounds, namely ferritin and non-crystallizable ferritin; and a microscopically visible granular iron material, hemosiderin. The latter, hemosiderin, »appears generally in the marrow, spleen and liver after rapid hemoglobin destruction or rapid and excessive iron deposition».

»Cook (1929) and Asher (1933) have examined these granules of horse spleen and found them to consist of protein material containing brown iron hydroxide and small amounts of phosphate and calcium.» »These granules may vary in composition from relatively little proteinaceous material and high iron content to relatively low iron content.» Otherwise the properties of hemosiderin and its significance to the iron metabolism are still unknown.

»The colloidal iron compounds, ferritin and non-crystallizable ferritin, appear to contain iron associated with one or possibly more proteins. The iron is in the form of reddish-brown ferric hydroxide of the approximate composition $(\text{FeOOH})_8(\text{FeOPO}_3\text{H}_2)$, the iron being uniquely characterized by magnetic measurements as possessing a constant magnetic dipole moment of 3.8 Bohr magnetons, a moment rarely observed in other iron compounds.»

»When ferritin iron micelles are attached to a specific protein, apoferritin (through an unknown chemical linkage), the complex of this protein with the ferritin-iron micelles is designated as ferritin. Apoferritin is a homogeneous protein which may be readily crystallized with cadmium sulphate, yielding colorless crystals. When apoferritin has iron micelles attached, then red-brown crystals of ferritin may be obtained with a maximum amount of iron equivalent to as much as 23 per cent of the dry weight of the dialyzed crystalline material.»

»Some of the ferritin-iron micelles appear to be attached to proteins which cannot be crystallized. These inhomogeneous materials, which still possess the characteristic ferritin-iron micelles, are placed together into a group termed the 'non-crystallizable ferritin'.»

For ferritin formation not only should the cell be able to produce apoferritin but it must have a mechanism for accumulating iron and possibly another for attaching iron to apoferritin. These conclusions are suggested by the observation that apoferritin, but little or no ferritin, is present in the testicles.

Granick, Hahn *et al.* (1943—44) have made a number of observations which lend support to the view that ferritin is an iron-storage compound. Thus a dog was given an injection of red blood cells which contained radioactive iron as a constituent of the heme in hemoglobin, and then intravital hemolysis was induced in the dog by injection of acetylphenylhydrazine. The dog was killed after 6 days, and ferritin was prepared from its liver. In the crystallized ferritin every four iron atoms were radioactive. — After intravenous injection of radioactive ferriammonium citrate into a dog, which was killed after 13 days, 80 % of the iron was recovered from the liver. Three-fourths of the iron in the crystallized ferritin was radioactive. The rate at which intravenously injected iron is transformed into ferritin

iron could be demonstrated to be very high. As early as 1 hour after the injection 40 % of the radioactive iron was recovered from the liver, where it was a constituent of the ferritin molecules.

Other experiments showed that »in anemic animals there was a drain not only of iron but also of apoferritin and probably of other proteins to meet the demands of rapid blood formation».

»Recently a marked increase of ferritin in the gastro-intestinal mucosa was observed in response to iron feeding.» »No apoferritin crystals could be detected before iron feeding, indicating that apoferritin was not originally present in any appreciable concentration. These facts suggest that the feeding of iron has in some way brought about an increase in the concentration of a particular protein which combines with iron to form a storage compound of iron in the mucosa» (Granick 1946).

Serum Iron.

After the pioneering works — above all, by Fontés & Thivolle (1925), Barkan (1927), Henriques & Roche (1927), Warburg (1927), Warburg & Krebs (1927) — through which the existence of serum iron was established, its variations in pathologic and physiological conditions have been the subject of thorough studies.

Serum iron is defined as acid-soluble iron in the serum, i.e., iron present in the serum as a constituent of a high-molecular complex from which it may be obtained in ionized form by simply acidifying the serum with hydrochloric acid. Hemoglobin is an iron complex that is more difficult to split. Normally the serum contains practically no ionized iron.

The main results of experimental and clinical serum iron research are given in monographs by Heilmeyer & Plötner (1937), Skouge (1939), Vahlquist (1941), Büchmann (1941) and Vannotti & Delachaux (1942).

Additional knowledge concerning the serum iron has been obtained through studies with radioactive iron — in particular by Hahn *et al.* (1939—47). The serum iron is generally accepted as the transport iron fraction.

The view advanced by Barkan (1937—39) — that the red blood cells contained an iron fraction which may be given off to the

plasma gradually as the red cells are getting older — is not accepted by more recent authors (Moore, Minnich & Welch 1939, Venndt 1940, Miller & Hahn 1940), who have shown that this iron fraction at least in part is a technical artefact. After extensive clinical studies Moore, Minnich & Welch arrived at the following conclusion: »The physiological importance of this blood iron fraction ('easily split-off') has fairly definitely been divorced from the function of iron transportation».

Variations of Serum Iron.

Normally the serum iron concentration is about $120 \gamma \%$ (80—200). Reckoning the plasma volume of a normal adult person as about 3.5 liters, the transport iron fraction thus amounts to about 4 mg. iron. The transport of iron from erythrocyte-destroying organs to erythrocyte-forming organs is so efficient that the iron liberated in the destruction of hemoglobin is utilized practically quantitatively for the synthesis of hemoglobin (Cruz, Hahn & Bale 1942). The high rate of the serum iron exchange is evident from the following calculation: The total amount of hemoglobin includes about 2.5 g. iron; and the lifetime of the red blood cells is given by Singer & Weisz (1942) as 3—4 months at the most. *So the amount of iron required daily for the normal hemoglobin synthesis is at least 25 mg., that is, 6 times larger than the entire amount of serum iron.*

The iron atoms move continuously in a circle: hemoglobin — (storage iron) — serum iron — (storage iron) — hemoglobin. On this account the serum iron level signifies a dynamic equilibrium between the destruction and the new-formation of hemoglobin, the storage iron serving as a buffer in the system.

The clinical observations of the variations of serum iron may be presented schematically as follows:

Increase in serum iron is obtained in

a) morbid conditions where the erythrocyte destruction is more rapid than the new-formation: Hemolytic crisis, pernicious anemia, polycythemia in remission (X-ray treatment), aplastic anemia, processes reducing the amount of bone marrow;

- b) hepatitis at the initial stage (destruction of the depot?);
- c) administration of iron, perorally or intravenously;
- d) («Physiologische Hypersiderämi») (Vahlquist).

Decrease in serum iron is obtained in

- a) conditions where the new-formation of red blood cells is more rapid than the destruction: anemia in regeneration, polycythemia in progression;
- b) sideropenia (with or without anemia);
- c) increased iron requirement in non-erythropoietic organs : malignant neoplasms, acute and chronic infections, artificial hyperthermia (after injection of animal or synthetic toxins);
- d) increased iron output, *e.g.*, in nephrosis (combination of infection and loss of iron), lactation, latter half of pregnancy.

Serum Iron and the Iron-binding Capacity of Serum.

With a view to the keystone position of the serum iron protein compound in the intermediary iron metabolism, the establishment of its chemical and physical characters is of the greatest interest. »Barkan (1927) found *serum iron non-dialyzable* at the pH of the blood. He (1933) further found that in native serum the serum iron is not ultrafiltrable; only *when the serum was acidified did iron pass into the ultrafiltrate*. Vahlquist (1941) showed that serum iron is non-dialyzable within a range of pH from 4.5 to at least 10. Vahlquist's dialysis experiment in vivo also supported the view that serum iron is bound to proteins. Barkan & Schales (1937) found that *half-saturation with ammonium sulphate precipitated serum iron* quantitatively together with the globulins. Such was also observed to be the case with sera to which iron has been added (Starkenstein & Harvalik 1933). With the aid of modern electrophoresis technique Vahlquist (1941) has made a small series of experiments regarding the binding conditions of serum iron. His results are difficult to judge of, as the iron concentrations in the analyzed fractions often were very low, the experiments were performed at different pH, and the iron concentration of the buffer employed varied. His conclusions are that serum iron is bound both to the albumin and the globulin

fractions. The main part is found in the last mentioned fractions, and one experiment seems to indicate that the α - and β -globulin fractions serve as the principal carriers, as the γ -globulin fraction is poor in iron» (Holmberg & Laurell 1945).

More recently experiments on purification of the iron-binding protein fraction in serum have been carried out in U.S.A. and also in the laboratory of the Medical Clinic, University of Lund.

The American authors (Oncely, Scatchard & Brown) have published a tabulary preliminary report on the physicochemical characters of some purified serum constituents (Advances of Protein Chemistry III: 460, 1947). The iron-binding serum component is taken to be identical with a β_1 -globulin fraction (IV: 7), but so far it has not been practicable to establish this.

In experiments on the purification of the iron-binding protein component of serum we have been able in the Lund laboratory to isolate this fraction in a state that is uniform on ultracentrifuging and on electrophoresis. The iron-free fraction is colorless, but the iron-containing complex is intensely red in color. It is not precipitated by half-saturation with ammonium sulphate (pH 8—5). Its molecular weight is found to be about 88,000, its isoelectric point is about pH 4.5. A detailed report of these studies will be published within a near future in Acta Chem. Scand. (in cooperation with B. Ingelman).

The factors influencing the serum iron concentration, which normally varies between about 80 and 200 γ ‰, are known but slightly. In order to get some substantial idea about the regulatory mechanism attempts have been made to influence the serum iron concentration in normal subjects and patients by oral and parenteral administration of iron. The results obtained in peroral tolerance tests proved very difficult to interpret, as the rate of absorption as well as elimination from serum were subject to wide individual variation. From the literature it is evident, however, that the serum iron concentration after ingestion of 0.25—1 g. ferrous or ferric iron usually lies at a level of 200—300 γ ‰. It appears as if values over 400 γ ‰ are not obtained in normal subjects.

Skouge (1939) and Waldenström (1944) performed tolerance tests with intravenous injection of 10 mg. iron and found the serum iron value, determined 5 min. after the injection, to be lower than was to be expected if the iron had been distributed on the entire plasma volume of the organism. Waldenström (1944) and Brochner-Mortensen (1943) were able to show that when

untreated patients with pernicious anemia were given 10 mg. iron intravenously the result was often merely an insignificant rise in serum iron. Further, Waldenström gave a normal subject two intravenous injections of iron at a short interval and found the rise in serum iron to be much smaller after the second injection than after the first.

From Waldenström's material it is also evident that symptoms of intolerance after the injection (flushing to the face, nausea, sneezing and headache) appeared in those patients who showed only a slight rise in serum iron after intravenous injection of iron. Such toxic symptoms appeared also in normal subjects when more than 10 mg. iron was injected. From these findings Waldenström arrived at the conclusion that the rise in serum iron following intravenous injection of iron is broken after an individually variable limit for the serum iron concentration is reached. Iron injected after this »braking effect» has come into function is eliminated very quickly from the blood stream. As Waldenström was able to make sure that the iron was not absorbed by the formed elements in the blood, nor excreted with the urine, he took the braking effect to depend on some extravascular factor.

Holmberg then advanced the hypothesis that the braking effect was due to the circumstance that serum is capable of complex binding of iron in an amount corresponding to the serum iron value, and that iron injected after this border value for serum iron is reached is bound to the serum proteins in such a form that the compound readily is split *in vivo*. After experiments on enrichment of serum with iron *in vitro* combined with experiments *in vivo*, this hypothesis proved tenable (Holmberg & Laurell 1945).

For the latter authors were able to show *that the iron in the native serum iron protein complex at physiological pH did not react with $\alpha\alpha_1$ -dipyridyl (reagent for Fe^{++}) in spite of the presence of sodium hydrosulphite. On enrichment of the serum with Fe^{++} also the iron added was bound, up to a certain border value (saturation limit), in non-dipyridyl-reacting form. On the other hand, all iron added over the saturation limit reacted with dipyridyl. On determination of the saturation limit in 10 normal subjects an average value of 312 γ % was obtained, which agreed*

very well with the serum iron concentration of 291 γ % found by Waldenström after injection of 10 mg. iron into normal subjects.

On determination of the saturation limit and serum iron concentration in untreated cases of pernicious anemia, Holmberg & Laurell (1945) found the saturation limit of serum most often to be equal to the serum iron level or a little higher. Intravenous injection of iron into these patients was followed by symptoms of intolerance even after a couple of milligrams. In two patients the serum iron level and the saturation limit were determined both before and after the institution of liver therapy. Thus the saturation limit was found not to be influenced by the liver therapy during the first days even though serum iron fell from high values to low. From their experiments the authors arrived at the conclusion that the braking effect is an expression of the ability of the serum to bind iron firmly and that administration of iron in excess of this capacity leads to the appearance of toxic symptoms.

Schade & Caroline (1944) were able with a microbiological method to demonstrate a protein component in raw eggwhite capable of binding iron. Subsequently this was purified by Alderton, Ward & Fevold (1946).

With their microbiological method Schade & Caroline (1946) found also human plasma to contain an iron-binding component. In plasma fractionated after Cohn *et al.* (1945) the greater part of the iron-binding component is found in fraction IV-3.4 (rich in α - and β -globulins) which is quite in keeping with the observation made by Vahlquist (1941): that after electrophoretic separation of serum the iron is found chiefly in the α - and β -globulin fraction.

Schade & Caroline determined the iron-binding capacity of serum in a normal subject as 260 γ %.

The iron-binding component in plasma determined by Holmberg & Laurell (1945) appears to be identical with the one determined by Schade & Caroline (1946) as is evident from the following observations.

Serum thus contains a protein component that is able to bind iron (supplied in vivo or in vitro) in a form that is identical with or rather like the binding of iron by the native serum iron protein complex.

The saturation limit of serum for iron signifies the capacity of the serum for firm complex binding of iron, and includes both the native iron content of the serum (=serum iron=manifest iron-binding capacity) and the increase in the concentration of firmly bound iron in the serum (=latent iron-binding capacity) resulting from the supply of iron to the serum.

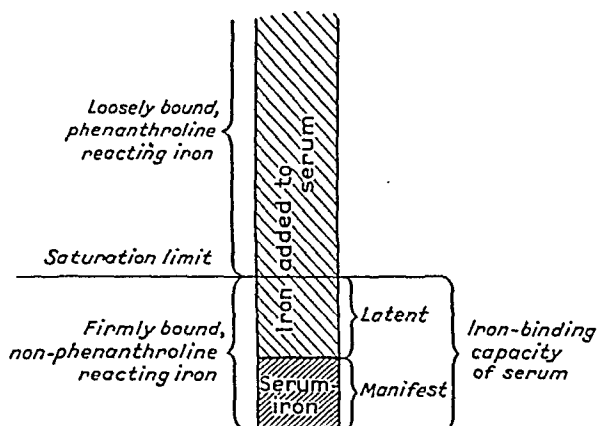


Fig. 1. Graphical presentation of terms employed.

EXPERIMENTAL INVESTIGATIONS.

Chapter I.

Methodics.

Determination of Serum Iron.

The method here adopted differs from the one employed by Holmberg & Laurell in that, instead of $\alpha\alpha_1$ -dipyridyl, *o*-phenanthroline is used as reagent for iron.

Technique.

1. *Cleaning of glassware.* — After being washed, all glassware to be used for the analysis is treated with dilute hydrochloric acid and then rinsed with glass-distilled water.

2. *Withdrawal of blood.* — For an analysis about 15 ml. blood is required. The blood is withdrawn by means of a stainless steel needle, polished inwardly, into a centrifuge tube.

3. *Analysis.* — Under shaking, 3 ml. 6N HCl is added dropwise to 6 ml. serum in a small Erlenmeyer flask. After 10 min., 6 ml. trichloroacetic acid is added. After additional 10 min. the mixture is filtered through a small Berzelius filter (the filter paper should be washed in 5 % HCl and rinsed with glass-distilled water, then dried). The filtrate is collected in a test tube.

Of this filtrate, 7.5 ml. is transferred into a volumetric flask of 12.5 ml., and 1 drop of paranitrophenol is added. Now ammonia is added, drop by drop till the indicator change in color is obtained. The mixture is then acidified with N/2 hydrochloric acid till the color has disappeared. After this, 0.2 ml. sodium acetate solution is added, and the sample is again acidified with hydrochloric acid, drop by drop, until the color disappears. Now 1 drop of phenanthroline solution and about 5 mg. sodium hydrosulphite are added. The mixture is diluted with glass-distilled water to 12.5 ml. and mixed well. The extinction value is read in a Pulfrich photometer against a blind that has been treated in the same way, containing distilled water instead of serum. The thickness of the layer should be 3 cm., the filter S 50. It is very important to make sure that the specimen is perfectly clear.

Calculation: The extinction value read is multiplied by 700, and this gives the iron content of the serum in γ per 100 ml. serum.

4. *Solutions required:*

6 N hydrochloric acid (conc., p.a. and equal parts of redistilled water).

20 % trichloroacetic acid (preferably redistilled).

Conc. ammonia.

N/2 hydrochloric acid.

1 N sodium acetate.

Paranitrophenol, 1 % solution in absolute ethanol.

Phenanthroline, about 2 % solution in 10 % ethanol.

Sodium hydrosulphite ($\text{Na}_2\text{S}_2\text{O}_4$) p.a. in substance.

Determination of the Iron-binding Capacity (Saturation Limit) of Serum.

In this chapter an account will be given of 3 different methods by which the iron-binding capacity of the serum may be determined. Methods A and B, which were first worked out, were not employed in the routine analyses. Method C is the simplest and most reliable of the three; in addition it requires less serum than the two others. So it has been adopted as routine method. Still methods A and B will also be described here in detail, as they are of value, for one thing, in experiments on purification of the iron-binding component of serum.

Method A.

This method is the one originally given by Holmberg & Laurell for determination of the saturation limit. As some minor changes had to be made for the sake of greater exactness, the method is given here in extenso, including these changes.

Principle. — In determination of the total amount of iron that may be firmly bound (non-phenanthroline-reacting) by a given serum, a known amount of iron is added, so that the total amount of iron in the serum exceeds the saturation limit. The loosely bound phenanthroline-reacting iron is determined colorimetrically. The saturation limit is found by subtracting the value for phenanthroline-reacting iron from the total amount of iron in the serum (after the addition of iron), both values being given in γ %.

Cleaning of glassware. — The same procedure as described above under total serum iron. About 40 ml. blood is required.

Analysis:

I. Determination of serum iron.

II. 4 ml. serum is pipetted into each of two test tubes. 0.25 ml. ferrous chloride solution is added to one tube, 0.25 ml. ascorbic acid solution to the other tube. After 10 min. the two samples are read against each other in a Pulfrich photometer with filter S 50, in 1 cm. cuvettes. The difference in extinction is noted (E_1). Some crystals of phenanthroline are added to the tube containing ferrous chloride. During the following 10 min. the tubes are shaken repeatedly; then about 5 mg. sodium hydrosulphite is added to either tube. After 1 hour they are read once more against each other with the same filter and thickness of layer as before (E_2).

Calculation:

$$\text{Saturation limit} = \text{serum iron} + 625 - \frac{(E_2 - E_1) \times 504 \times 4.25}{4}$$

(in γ ‰)
(in γ ‰)
(Fe addition
in γ ‰, on
employment
of 0.25 ml.
Fe standard)
(phenanthroline iron
in γ ‰ as iron)

Reagents:

Chemicals required for serum iron determination.

Ferrous chloride solution, made from a stock solution of ferric chloride (100 mg. Fe and 1 ml. conc. HCl per liter water).

10 ml. stock solution is reduced with 20 mg. ascorbic acid, immediately before using.

Ascorbic acid solution: About 20 mg. ascorbic acid in 10 ml. water.

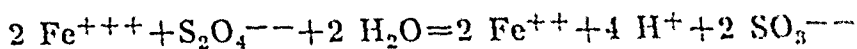
Discussion.

1. In the original method the serum under analysis is kept in a test tube with a gas mixture containing 5 ‰ CO_2 and 95 ‰ N_2 in order to keep the serum pH as near the physiological pH range as possible. In page 50 an account is given of the influence of shifts in pH on the iron-binding capacity of the serum. From the findings there recorded it is evident that in determination of the saturation limit the serum pH should fall within the range of

8—7.3. With the method here employed, on the acid side of pH 7, the iron-binding capacity of the serum falls rapidly.

Both the addition of the acid iron standard and of sodium hydrosulphite shift the serum pH in acid direction.

Sodium hydrosulphite reduces Fe^{+++} after the formula:



The amount of hydrosulphite required for quantitative reduction of the loosely bound iron after addition of a ferrous or ferric iron compound was determined in an experiment recorded in Table 7 (p. 29). From this experiment it is evident that an addition of 5 mg. sodium hydrosulphite is quite sufficient to reduce the loosely bound iron in 4 ml. serum enriched with about 500 γ % iron — regardless whether the iron is added as a ferrous or a ferric compound.

The shift in serum pH after addition of the iron standard and 5 mg. sodium hydrosulphite amounts to about 0.4. If the saturation limit is determined on a freshly centrifuged serum, before the carbon dioxide is all given off, the determination will thus be made with the serum pH of about 7, and the result obtained from the analysis will be somewhat too low.

In order to standardize the experimental conditions the sera were generally frozen to a temperature of about -20° and not analyzed till the following day. On thawing, at room temperature the serum has a pH of 8—8.2, and thus the pH resulting after the addition of hydrosulphite and iron will be about 7.7, i.e., falling within the suitable range of pH. Keeping the serum specimens at the aforementioned low temperature offers also the advantage that the analysis may be postponed even for a week or more without influencing the result.

2. On addition of iron to a serum of high latent iron-binding capacity the serum color (E_1) changes so that the light adsorption increases in the blue field of the spectrum. On addition of iron to a serum of no latent iron-binding capacity the color changes but quite slightly or not at all.

In their calculation of the amount of iron reacting with phenanthroline, Holmberg & Laurell found it justifiable to subtract E_1 from E_2 .

Subsequently Schade & Caroline (1946) observed »a typical salmon pink colour» in the serum after addition of iron — just

as they (1944) had found the white of hen's egg to be colored pink, as it specifically binds Fe^{++} ions. Like Holmberg & Laurell, they found the increase in color (E_1) to appear successively on the addition of very small amounts of iron and to attain its maximum at the saturation limit. It has been possible to establish that the correct value for the phenanthroline iron formation is obtained by subtracting E_1 from E_2 . For when the phenanthroline iron is removed from the serum by adsorption with activated charcoal (see p. 17), there still remains the color difference E_1 between the serum enriched in iron and the control.

Holmberg & Laurell and Schade & Caroline found that the increase in serum color after addition of ferrous iron attained its highest value when iron was added up to the saturation limit.

The writer investigated therefore whether the change in serum color after addition of iron might be directly proportional to the latent iron-binding capacity of the serum. In 40 sera from normal subjects and patients the serum iron was determined as well as the saturation limit (after method C) and the increased extinction value (E_1) after enrichment of the serum with ascorbic acid-reduced FeCl_3 above the saturation limit. E_1 was measured in the way described on p. 14. The results are recorded in Fig. 2.

In Fig. 2 the increase in serum color after addition of iron is plotted on the ordinate, the latent iron-binding capacity along the axis of abscissas. From this figure it is evident that *a single determination of the change in serum color after iron enrichment gives an approximate expression of the latent iron-binding capacity of serum*. With a normal or low latent iron-binding capacity the increase in serum color gives a very uncertain value for the latent iron-binding capacity. Presumably this is due to the circumstance that the iron-ascorbic acid solution added does not react alone with the iron-binding compound but also with the other colored components of the serum.

Serviceability of Method A.

Subsequently more simple methods have been worked out for determination of the iron-binding capacity of the serum (methods B and C). For particular purposes, however, method A may still be preferable. In estimation of the iron-binding capacity, for instance, of hen's eggwhite after method

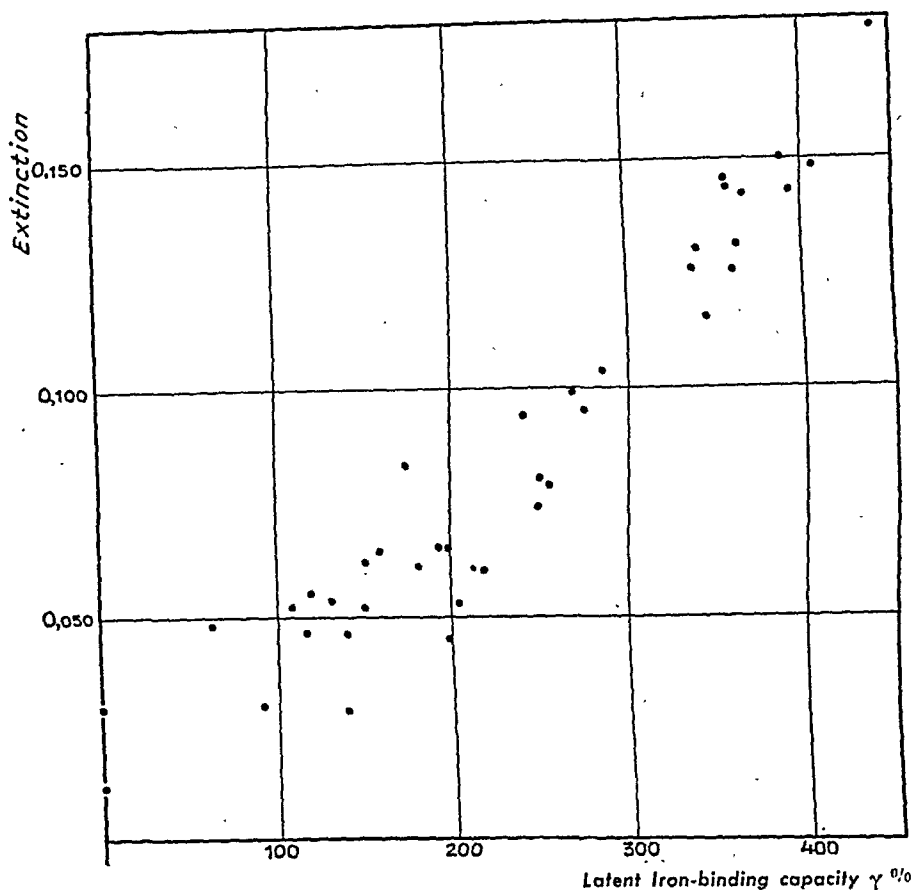


Fig. 2. Relation between the change in serum color (read with filter S 50 and cuvette layer 1 cm.) on administration of iron and the latent iron-binding capacity of serum.

B or C it is difficult to obtain a clear filtrate after precipitation of albumin with trichloroacetic acid; and this makes the results highly uncertain. On the other hand, the latent iron-binding capacity of raw eggwhite may readily be determined after method A.

Method B.

Freundlich & Birstein (1927) have shown that the ferrous salts of *o*-phenanthroline and $\alpha\alpha_1$ -dipyridyl are adsorbed by activated charcoal. The adsorbent affinity is almost just as high as for aniline and phenol.

On the basis of this observation a new method has been worked out for determination of the iron-binding capacity of serum.

Principle. — Iron is added to the serum; then phenanthroline and sodium hydrosulphite are added. Phenanthroline is allowed to enter into a complex compound with the loosely bound iron, the mixture being left standing for 1 hour — just as in method A. Then the phenanthroline iron is adsorbed with activated charcoal. After this adsorption only non-phenanthroline-reacting iron remains in the serum. Then the value for the saturation limit is obtained by means of an ordinary assay of the iron present in the serum after the adsorption.

Performance of Assay. — To 5 ml. serum (pH ca. 8) is added 0.25 ml. ferrous iron solution (100 γ /ml.). Then a dozen phenanthroline crystals are added to the mixture, in which they dissolve under repeated shaking in the following hours, whereafter about 5 mg. sodium hydrosulphite is added. The mixture is left standing for 1 hour, whereafter 100 mg. charcoal is added. After repeated shaking during the following 5—10 min., the charcoal is separated from the serum by centrifuging or filtration. Finally, in ordinary serum iron determination is performed on 4 ml. of the adsorbed serum, which is free from phenanthroline as well as phenanthroline iron.

Calculation: $1045 \times \frac{4.25}{4} \times \text{extinction} = \text{the amount of iron } (\gamma)$
bound by 100 ml. serum.

Discussion.

The adsorbent here employed was *carbo activatus* (für Urinanalyse [Merck]). The iron content of the charcoal, determined after ashing, was about 0.3 %. In order to cut down this iron content, the charcoal was boiled with hydrochloric acid.

In the first experiments the values obtained for the saturation limit were generally too high as compared with the values obtained after method A. This was difficult to explain, as the serum color after the adsorption indicated that no phenanthroline iron remained in the serum. Furthermore, by control tests it could be established that the serum did not absorb any iron from the charcoal even though this was not iron-free.

Adsorption experiments with phenanthroline iron in aqueous solutions.

Charcoal was added to a solution of phenanthroline iron in phosphate buffer (pH ca. 7), after which the solution soon was decolorized. After removal of the charcoal by centrifuging the solution was perfectly colorless. But when now phenanthroline was added to the clear solution, the color partly returned, due to the formation of phenanthroline iron. Thus it was obvious that *free iron ions appeared in the solution when the phenanthroline iron was adsorbed on charcoal*. This was rather surprising, as phenanthroline iron is stated to be a most stable complex at neutral or slightly acid pH.

In order to establish that the free iron ions which appeared in the solution after adsorption of phenanthroline iron did not originate from iron impurities in the charcoal, this was rid of iron as far as possible.

But it was difficult to lower the iron content of the charcoal as far as desirable. This iron in the coal showed optimal solubility in 1—2 N HCl. In order to lower the iron content in a desirable degree the charcoal had to be boiled with the acid for two months (the acid was removed by filtration and replaced by fresh acid twice a day). After this, the charcoal was neutralized by repeated boiling with dilute ammonia. Finally the charcoal was boiled repeatedly with water and a weak phosphate buffer (pH 7) till the water showed neutral reaction.

The iron content of the charcoal purified in this way was about 3 γ iron/100 mg. charcoal. The adsorbent affinity of this charcoal for phenanthroline and phenanthroline iron was high, notwithstanding its protracted treatment (see Fig. 3).

From Fig. 3 it is evident that phenanthroline is more adsorbable on charcoal than is phenanthroline iron. Indeed, this was also to be expected, as phenanthroline iron is more water-soluble than phenanthroline.

Now the experiments were repeated with the purified charcoal in order to ascertain where the free iron ions originated from. The experimental results are given in Table 1, illustrating how phenanthroline iron is dissociated in part when charcoal is added to a solution of phenanthroline iron. In this experiment the

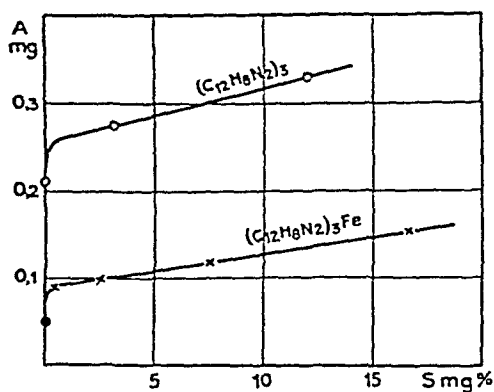


Fig. 3.

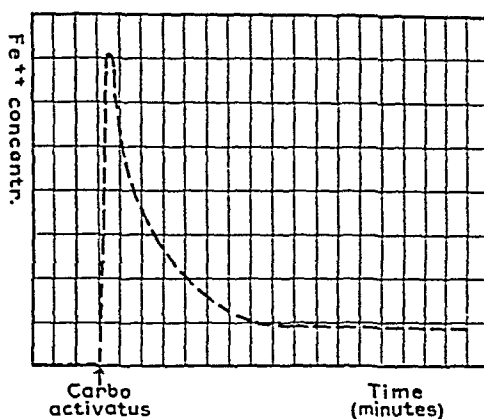


Fig. 4.

Fig. 3. Adsorption of phenanthroline and phenanthroline iron by activated carbon. Volume of solution: 4 ml. Activated carbon employed: 10 mg. A: Quantity adsorbed at adsorption equilibrium. S: Concentration in the bulk of the solution at adsorption equilibrium. The values are given as the amount of iron equivalent to phenanthroline.

Fig. 4. Changes in the iron concentration of the phenanthroline iron solution on addition of activated carbon.

phenanthroline iron concentration was of the same magnitude as in the determination of the saturation limit on serum.

An aqueous solution (II) of phenanthroline in M/15 phosphate buffer after Sørensen (pH 6.5) was diluted so that exactly 1 ml. was required for the complex binding of 1 ml. iron standard (Fe^{++}). Another phenanthroline solution (I) was prepared by diluting solution II ten times. 0.4 ml. Fe^{++} standard was mixed with 4 ml. of solution I in a series of tubes. The solution obtained was thus practically free from ferrous ions as well as phenanthroline. Increasing quantities of phenanthroline in excess were added in the form of solution II. The last tube was saturated with phenanthroline crystals. Then the solution in each tube was adsorbed on charcoal. After shaking the mixture with charcoal for 10–15 min., the coal was separated by centrifuging. After this, the solution was treated with phenanthroline crystals and with sodium hydrosulphite, so that any free Fe^{++} ions would react with the phenanthroline. The quantity of iron liberated by the adsorption could later be determined colorimetrically. Prior to the adsorption, all the specimens contained phenanthroline iron corresponding to 645 γ % Fe.

In the experiment described, complete absence of color was obtained in all the tubes with 25 mg. and 100 mg. charcoal. With 10 mg. charcoal, only the first two tubes were colorless after the

adsorption. With an excess of phenanthroline (II=0.4, 0.8, 1.6 and crystalline phenanthroline) the following concentrations of non-adsorbed phenanthroline iron were obtained: 60, 124, 210, 338 % (phenanthroline iron reckoned as iron).

Table 1.

Formation of Fe^{++} ions on addition of activated charcoal to a solution of phenanthroline iron $[(C_{12}H_8N_2)_3Fe]^{++}$

Fe ⁺⁺ standard ml.	Buffer		H ₂ O ml.	Fe ⁺⁺ concentration in solution after adsorption		
	I. ml.	II. ml.		10 mg. coal γ %	25 mg. coal γ %	100. mg. coal γ %
0.4	4	0	1.6	89	90	90
0.4	4	0.2	1.4	56	53	24
0.4	4	0.4	1.2	40	30	0
0.4	4	0.8	0.8	11	—	—
0.4	4	1.6	0	4	0	0
0.4	4	Phen. cryst.	1.6	(12)	0	0

From Table 1 it is evident that the appearance of free iron ions in the solution after adsorption of phenanthroline iron can be prevented by addition of phenanthroline in sufficient excess. The greater the amount of charcoal employed, the lesser excess of phenanthroline will be required. With 10 mg. charcoal the adsorbent substance is not sufficient to eliminate the phenanthroline iron from the solution in the presence of phenanthroline in excess.

Fig. 4 shows schematically how the Fe^{++} ion concentration varied when activated charcoal is added to a solution of phenanthroline iron. The concentration of Fe^{++} ions reaches its maximum within the first minute after addition of the charcoal. Later on, the Fe^{++} concentration falls again, reaching a practically constant value after 10—15 min. The declining limb of the curve represents a new complex formation between the adsorbed phenanthroline and the Fe^{++} ions in the solution. The greater the excess of phenanthroline in the phenanthroline iron solution, the more will the curve be flattened.

This dissociation phenomenon, I think, is due to the circumstance that phenanthroline is adsorbed more readily on charcoal

than is phenanthroline iron (Fig. 3). Thus the dissociation equilibrium in the system $(C_{12}H_8N_2)_3Fe^{++} \rightleftharpoons Fe^{++} + 3 C_{12}H_8N_2$ shifts from left to right when the charcoal is added. This may practically be prevented completely, however, by addition of phenanthroline in excess prior to the charcoal adsorption.

Adsorption of Phenanthroline Iron from Serum.

When an iron-saturated serum containing phenanthroline iron is treated with charcoal, this will adsorb the phenanthroline iron. The iron content of the serum after the adsorption will be equal to the saturation limit if the serum contains an excess of phenanthroline $\left(\frac{3(C_{12}H_8N_2)}{(C_{12}H_8N_2)_3Fe} > 5 \right)$. If the experiment is performed without the addition of phenanthroline in excess, after the adsorption the serum will still contain some loosely bound iron.

If, on the other hand, charcoal is added to a native serum enriched with phenanthroline iron, the iron-binding compound will take up the iron ions liberated by the adsorption and prevent them from being bound again by the adsorbed phenanthroline. On adsorption, therefore, the iron content of the serum increases to a concentration that corresponds to the value for the saturation limit. Even by working with a very large excess of phenanthroline it is not possible in this case to prevent the iron-binding compound from taking up some iron, *i.e.*, the serum iron will increase.

Method C.

This was the routine method employed in the present studies. If nothing else is stated explicitly, all determinations reported in the following were performed after this method.

Principle. — The first steps of the analysis are carried out after the same principle as in methods A and B.

I. The serum is enriched with an iron salt. On addition of phenanthroline and sodium hydrosulphite the loosely bound iron enters into the complex phenanthroline iron.

II. The serum iron determination is made directly on the serum containing a mixture of protein-linked iron and phenanthroline iron. The iron content of the filtrate after precipitation of protein

gives an expression for the protein-linked iron — owing to the following facts, which will be analyzed later on:

a) On addition of 6 N hydrochloric acid to serum, the protein-linked iron is dissociated and becomes filtrable.

b) On addition of trichloroacetic acid the phenanthroline iron is precipitated.

c) At room temperature the phenanthroline iron dissociates but very slowly, in spite of the acid milieu. When it is precipitated, its dissociation rate is even more slow.

d) Under the given conditions no phenanthroline iron is formed anew in the strongly acid milieu at any measurable rate.

Performance of Assay. — Serum free from hemolysis that has been stored at -20° is thawed and mixed thoroughly.

4 ml. serum is pipetted into each of two test tubes. To one tube is added 0.15 (0.2) ml. — to the other tube 0.25 (0.3) ml. — of ascorbic acid-reduced ferric chloride. To the first tube 0.1 ml. water is added — to make the same volume in the two tubes.

To either tube is added 0.2 ml. phenanthroline solution and about 5 mg. sodium hydrosulphite. After thorough mixing, the specimens are left standing for 1 hour.

In two 50 ml. flasks, 2 ml. water is mixed with 3 ml. hydrochloric acid (6 N) and — after one hour — 4 ml. of the serum mixture is allowed to run down slowly into each flask under cautious shaking, so that a fine floccular precipitate is obtained. After 10 min., 6 ml. trichloroacetic acid is added; and the mixture is filtered after additional 10 min.

7.5 ml. of the filtrate is transferred to a 12.5 ml. volumetric flask and neutralized with ammonia till color reaction of para-nitrophenol. 0.2 ml. sodium acetate is added, whereafter the mixture is acidified with N/2 HCl, until the yellow paranitrophenol color disappears, and the pure phenanthroline iron color is obtained.

Now 0.5 ml. phenanthroline solution and about 5 mg. sodium hydrosulphite are added, and the flask is filled with redistilled water to the 12.5 ml. mark. The contents are mixed at once in order to avoid any local excess of acid and hydrosulphite in the neck of the flask.

After 30 min., at the earliest, the color of the specimen is read against a control which is made up in the same way as the control for serum iron determination.

The results were read in a Pulfrich photometer, with a layer of 3 cm., and with filter S 50.

Calculation: $1045 \times \frac{4.45}{4} \times \text{extinction} = \text{the amount of iron } (\gamma),$
bound by 100 ml. serum.

If the determination has to be made on a smaller amount of serum, it is more suitable to use 5 cm. microcuvettes for the reading. This requires only an end-volume of 1 ml., and thus the analysis may be carried out on less than 1 ml. serum.

Reagents. — The same reagents are used as for determination of serum iron. Saturated aqueous phenanthroline solution.

Discussion.

In its *first stage*, the method is based on the same principle as methods A and B. Iron is added to the serum, and the iron ions which are not bound specifically to the serum are linked into the phenanthroline complex. As will be pointed out in detail later on (Chapter II, p. 48), during the development of the color reaction the serum pH has to be kept between 8 and 7.5, as a lower pH will give too low values. Freshly centrifuged serum (or plasma) has pH ca. 7.5—8.2, depending on the amount of carbon dioxide given off under the centrifuging. The determination cannot off-hand be made on a serum with pH 7.5, as the addition of iron standard and hydrosulphite for reduction of the loosely bound iron gives a shift of pH in acid direction. The extent of this acidification will depend primarily on the amount of hydrosulphite (Table 2).

In order to standardize the experimental conditions as far as possible, all the serum specimens have been frozen at -20° and stored for 24 hours before their analysis. As a result of this, carbon dioxide has been given off so that the serum pH after thawing has been about 8.2. Storage at frozen state prevents any bacterial growth in the serum, and its iron-binding capacity keeps unchanged at least for one week.

Table 2.

Changes in serum pH on addition of sodium hydrosulphite.

Sodium hydrosulphite added to 4 ml. serum mg.	pH
0	8.25
5	7.95
10	7.5
25	7.0

In the *second stage of the analysis* the protein-linked iron is ionized by addition of 6 N HCl. The protein and phenanthroline iron are precipitated with trichloroacetic acid, so that filtration of the mixture gives a filtrate free from protein and phenanthroline iron. The iron content of the filtrate thus corresponds directly to the amount of iron bound by serum in a non-phenanthroline-reacting, acid-soluble form. This statement is based on observations made on reactions of phenanthroline and phenanthroline iron in acid milieu.

1. In a mixture of serum, phenanthroline and phenanthroline iron, both the phenanthroline and the phenanthroline iron are easily dialyzable. When the serum is acidified with 6 N hydrochloric acid, the protein is precipitated in part, adsorbing partially both phenanthroline and phenanthroline iron. The pH of the solution is about 0. Phenanthroline iron constitutes a practically undissociated iron complex within the pH range of about 1.3—9. So, in the hydrochloric acid-serum mixture the phenanthroline iron ought to be dissociated. Blau (1898) has shown that phenanthroline iron is dissociated slowly in a weakly acid milieu. In a strongly acid milieu the reaction proceeds very rapidly on heating, but slowly when the mixture is kept cold. The rate of dissociation at room temperature is evident from Table 3, in which phenanthroline iron correlated with dipyriddy iron (stable within the pH range of 3—3.5 to 8.5).

6 ml. of the complex salts in aqueous solution are mixed with 3 ml. 6 N HCl. The extinction of the mixture was read after various lengths of time in 1 cm. cuvettes (filter S 50). As the dissociated complex is colorless, the extinction value is directly proportional to the amount of undissociated complex in the solution.

Table 3.

Dissociation rate of phenanthroline iron and of dipyridyl iron in 2 N hydrochloric acid.

Phenanthroline iron		Dipyridyl iron	
Time in min.	Extinction	Time in min.	Extinction
0	1.000	0	1.000
10	0.980	1	0.970
20	0.960	5	0.880
40	0.920	8	0.830
70	0.890	11	0.775
1440	0.210	20	0.640
		60	0.250

In method C the phenanthroline iron is exposed to the action of hydrochloric acid for 10 min. and to hydrochloric acid+trichloroacetic acid for 10—20 min. Experimental data show, however, that the dissociation of phenanthroline iron proceeds at a lower reaction rate in a mixture of serum and hydrochloric acid than in an aqueous solution of phenanthroline and hydrochloric acid. Presumably, the reason for this may be found in the following facts: a) serum acts as a buffer; b) the phenanthroline iron complex is adsorbed partially on the precipitated protein particles, so that the activity of the complex is lowered. As pH on the surface of the protein particles is more alkaline than in the main part of the solution, the conditions for dissociation are less favorable for adsorbed than for free phenanthroline iron.

By theoretical and experimental studies, Danielli (1942) has shown that »the pH at the surface of a protein molecule is different from that in the surrounding bulk phase». This phenomenon is associated with the »protein error» on determination of pH by means of indicators. »The indicator, if adsorbed, tends to give the pH of the surface phase and not of the bulk phase». If a protein is positively charged, the pH will be greater on the protein surface than in the bulk phase. In studies of ovalbumin Danielli found a difference of more than 1 pH unit between the surface phase and the bulk phase when pH of the medium was 2. So the chance of dissociation is more favorable for free than for adsorbed phenanthroline iron.

2. It is of the greatest importance to the serviceability of the method, however, to see that the *phenanthroline iron is precipitated on addition of trichloroacetic acid*. This applies also to

Table 4.

Saturation limit determined after method C with phenanthroline and with dipyridyl as complex builders of loosely bound iron.

Concentration of iron in serum γ ‰	Saturation limit (γ ‰) on analysis with	
	Phenanthroline	Dipyridyl
465	269	280
590	276	314
715	274	346
840	275	380

phenanthroline iron in aqueous solution, where it is precipitated as an amorphous powder. After 12 hours, needle-shaped brownish-red crystals are obtained. After precipitation of the complex, its dissociation proceeds but very slowly. Phenanthroline is not precipitated by trichloroacetic acid.¹ Dissociation of dipyridyl iron occurs in more acid solutions than pH 3. Table 4 gives some comparative results from determination of the saturation limit after method C, partly with phenanthroline as complex builder, partly with dipyridyl. From the data given it will be noticed that up to about 25 ‰ of the dipyridyl iron undergoes dissociation before the filtration is finished.

3. How the dissociation of phenanthroline iron in acid milieu is inhibited by cold is evident from the following experiment.

With employment of method C, up to about 1 ‰ of the phenanthroline iron is dissociated before the filtration is over. In order to be able to demonstrate the difference in the outcome, whether the analysis is performed at room temperature or at a low temperature, some experiments were carried out with about 100 times more phenanthroline iron in excess than usually. The serum was enriched partly (a) with about 200 γ ‰ Fe^{++} , partly (b) with about 12000 γ ‰ Fe^{++} over the saturation limit. Crystalline phenanthroline was added in excess to both serum portions. In each experimental series 2 specimens were treated with acid at room temperature, 2 at about 0°. All the specimens were filtered at room temperature. The results of the analysis were:

¹ Correspondingly, dipyridyl iron — but not dipyridyl — is precipitated on addition of trichloroacetic acid. Also the copper and zinc complexes of phenanthroline and dipyridyl are found to form crystalline compounds with trichloroacetic acid in acid milieu.

	Room temperature	About 0°
Serum (a)	293 γ %	295 γ %
" (b)	522 γ %	308 γ %

When the hydrochloric acid is added to the serum at 0° the dissociation of phenanthroline iron can be lowered to practically 0 within the period employed. From a practical point of view the dissociation is even so low at room temperature that it may be left out of consideration.

4. On addition of hydrochloric acid to the serum, the protein-linked iron is ionized. In method C this takes place in the presence of phenanthroline and hydrosulphite. The shift in pH on the acidification cannot take place instantaneously. So, conceivably some of the just ionized iron may have time to enter into complex-binding with the phenanthroline before the shift in pH passes 1.3, where the reaction is inhibited. If this were the case, the values obtained for protein-linked iron would be too low. In order to ascertain whether this might be the case, the following experiment was performed:

The serum iron content was determined in 2 series of tests. Phenanthroline was added to half of the specimens. Sodium hydrosulphite (5 mg.) was added to all the specimens. The results obtained are recorded in Table 5, from which the presence of phenanthroline appears not to have any influence on the outcome.

Table 5.

Serum iron γ % (Serum + hydrosulphite)	Serum iron γ % (Serum + hydrosulphite + phenanthroline)
186	189
188	189
180	182
214	218
211	214
209	212

Incomplete complex-binding may take place also at a pH lower than 1.3.

Therefore, the serum was saturated with phenanthroline in order to attain the optimal conditions for complex building in the acid medium.

Table 6.

Normal serum sample	A	B
serum iron γ 0/0	serum iron γ 0/0	serum iron γ 0/0
300	276	275
273	287	273
295	277	273

A) The serum saturated with phenanthroline was left standing with hydrochloric acid for 1 hour before trichloroacetic acid was added.

B) In this series also the trichloroacetic acid was added in the beginning, and the mixture was left standing for 1 hour. The serum had been enriched with iron below the saturation limit. Hydrosulphite was added to all the specimens. The results obtained are recorded in Table 6.

From these experimental results it is evident that no phenanthroline iron is formed when the serum is acidified with 6 N HCl in presence of phenanthroline.

5. A prerequisite of the method is that the amount of hydrosulphite added to the iron-enriched serum is sufficient for quantitative reduction of all the iron exceeding the saturation limit. The amount of hydrosulphite required for this was estimated in the following experiment (Table 7):

32 ml. serum was mixed with 1.6 ml. Fe standard (100 γ /ml.) and 1.6 ml. phenanthroline solution (saturated aqueous solution). The experiment was performed in two series of tests. In one the added iron was reduced with

Table 7.

Estimation of the addition of sodium hydrosulphite to a serum enriched with iron required for quantitative reduction of loosely bound iron.

Hydrosulphite added to 4 ml. serum mg.	Non-phenanthroline-reacting iron (γ 0/0) when iron was added as	
	Fe ⁺⁺	Fe ⁺⁺⁺
0	372	544
1	314	377
2.5	315	315
5	312	304
7.5	316	311
10	311	307

ascorbic acid (20 mg. ascorbic acid to 20 ml. Fe standard). In the other series the added iron was not reduced. In both series the serum contained about 540 γ % iron. Hydrosulphite was added in varying amounts. Before the addition of sodium hydrosulphite the serum pH was about 8. One hour after the addition of hydrosulphite, the amount of non-phenanthroline-reacting iron was determined.

If serum is enriched with iron to about 100 γ % over the saturation limit, 1 mg. hydrosulphite per 4 ml. serum appears sufficient to reduce this iron quantitatively. In order to make sure of a satisfactory margin of safety, however, a double amount of hydrosulphite has been used in the experiments.

Determination of the Saturation Limit in Hemolytic Sera.

Heilmeyer (1937) has shown that a moderate degree of hemolysis is of no consequence to the serum iron determination. The hydrochloric acid used in this determination liberates iron from the hemoglobin so slowly that the amount of iron thus liberated in 10 min. is quite negligible.

The amount of iron liberated from hemolyzed red blood cells by acid (easily split-off iron) was found to be a function of the acid, its concentration, time and temperature (Venndt 1940).

Under the action of acid, iron is liberated from carbon monoxide hemoglobin more slowly than from oxyhemoglobin (e.g. Venndt 1940).

When a solution of hemoglobin is acidified in the presence of a reducing substance (thioglycollic acid, hydrosulphite) the iron is liberated much more rapidly than in the absence of a reducing substance (Shorland & Wall 1936, Borgen & Elvehjem 1937).

When the saturation limit is determined on a strongly hemolytic serum, the values obtained will be higher than for the non-hemolytic serum. This is due to the circumstance that the iron in hemoglobin is bound in a non-phenanthroline-reacting form, and when the serum is acidified in the presence of a reducing substance, the hemoglobin iron will be ionized partially.

From carbon monoxide hemoglobin the iron is ionized far more slowly in acid milieu in the presence of a reducing substance than from oxyhemoglobin. The following experiments show how

it is possible to lower the experimental error by means of carbon monoxide in determination of the saturation limit and serum iron in hemolytic sera. These experiments were carried out with considerably larger amounts of hemoglobin than are obtained in ordinary sampling of the blood. (Heilmeyer [1937] designates even the presence of 30—40 mg. % hemoglobin in the serum as marked hemolysis.) This was done in order more readily to establish the influence of the hemoglobin.

a. Effect on hemolysis on the serum iron values obtained at determination in the presence of hydrosulphite:

60 ml. serum was divided into 3 portions, to two of which the hemoglobin solution was added (derived from washed hemolyzed erythrocytes).

Portion A contained native serum. It was saturated with CO.

» B » » » + hemoglobin. Saturated with CO.
» C » » » + »

All three portions were left standing with hydrosulphite for 1 hour, and then the serum iron content was determined in the usual way. The results are given in Table 8.

Table 8.

Effect of hemolysis on the serum iron values obtained on analysis in the presence of sodium hydrosulphite.

A Serum + CO γ % Fe	B Serum + Hb + CO γ % Fe	C Serum + Hb γ % Fe	Hemoglobin concentration mg. %
104	116	238	380
101	114	230	»
99	110	240	»

b. Effect of hemolysis on the saturation limit values:

Like the preceding, this experiment was performed with 3 serum portions, two of which were enriched with hemoglobin. The saturation limit was determined in the usual way. The results are recorded in Table 9.

When this experiment was modified in this way that the serum was not saturated with carbon monoxide till it had been standing with hydrosulphite for one hour (that is, immediately before it was acidified), this had no effect on the outcome.

As early as 1933 Barkan showed that saturation of the serum with carbon monoxide prior to the determination of its iron content has no influence on the values for serum iron. Thus it

Table 9.

Effect of hemolysis on the saturation limit values.

A Serum γ %	B Serum + Hb + CO γ %	C Serum + Hb γ %	Hemoglobin concentration mg. %
282	278	354	230
286	274	364	230
284	286	370	230

has been possible to confirm this observation and also that carbon monoxide has no influence on the results in determination of the saturation limit on a serum free from hemolysis.

Conclusion: The serum obtained from any specimen of blood can be demonstrated spectroscopically to contain some hemoglobin. The insignificant hemolysis usually present has no disturbing effect on the determination of the saturation limit for iron. Only when the hemolysis thus obtained is so strong that the naked eye distinctly notices an admixture of red in the usual serum color will the values for the saturation limit have a tendency to be somewhat too high, but any pronounced error in the determination appears only on analysis of strongly hemolytic sera. From the experimental results here recorded, however, it is evident that a determination of the saturation limit still is practicable if only the serum first is saturated with carbon monoxide.

Experimental error in determination of the saturation limit after method C.

From the results of 300 duplicate determinations on sera with varying saturation limit the experimental error was calculated to be $\pm 6 \gamma$ %. For various reasons, however, the experimental error obtained by means of duplicate determinations gives a somewhat too low value for the methodical error. The more important reasons for this are as follows:

a) The larger the amount of iron added in excess of the iron-binding capacity of the serum, the greater is the error of the determination. For, in the course of the analysis the loosely bound iron is transformed to phenanthroline iron, which later — when

the mixture is acidified — is dissociated in iron (up to about 1 %) and phenanthroline, making thus the value for the saturation limit somewhat too high.

b) The influence of the hemolysis is not reflected by duplicate determinations.

In the analysis, iron is always added in excess, and hence a systematic methodical error corresponding to 1 % of the phenanthroline iron formed is unavoidable. Of course, this error can be calculated in each individual case, but usually it does not amount to more than a couple of γ %, and therefore it has not been taken into account in the present work.

In determination of the saturation limit on clinical material duplicate analyses were carried out on every serum examined.

Other Analytical Methods Employed.

For determination of various other components of the blood the following methods have been used:

Hemoglobin was determined after Authenrieth & Königsberger (100 % Hb = 20.5 vol. % O₂).

Citric acid content of the serum was determined after the enzymatic method. Upper normal limit: 28 γ ml.

Phosphatases (alkaline) in the serum were determined after Buch & Buch (modified King-Armstrong method). Upper normal limit: 9 units.

Bilirubin in serum was determined after Jendrassik & Gróf. Upper normal limit: 1.3 mg. %.

Serum protein was determined after a modification of the Kjeldahl method for determination of nitrogen. The protein values given in Table 31 (p. 79) were obtained after Plimmer (modification of Van Slyke's copper sulphate method).

Serum albumin was estimated after precipitation of the globulins with saturated sodium sulphate at 37°.

Thymol, Turbidity test (MacLagan): The readings were made in a Pulfrich photometer. Upper limit of normal variation: 0.10.

Chapter II.

Aspects on the Linkage of Iron in Iron-enriched Sera.

Adsorption-analytical Determination of the Saturation Limit of Serum after Enrichment with Ferrous and with Ferric Salts.

The methods (A, B and C) described in the preceding chapter for determination of the iron-binding capacity of serum are all based on the circumstance that iron added to the serum in excess of the saturation limit reacts with phenanthroline in the presence of hydrosulphite. The value obtained in the saturation limit is the same whether the iron is added in a ferrous or ferric form. From this result it is not practicable, however, to decide whether the iron-binding protein in the serum may enter a complex linkage both with ferrous and ferric ions, or whether only ferrous ions may be taken up in this complex. This is due to the difficulty in deciding whether the iron in enrichment with a ferric salt is bound only after the addition of hydrosulphite, that is, when the ferric iron has been reduced. Still, there is evidence to the effect that the iron-binding protein is capable of complex linkage with ferric iron as well as ferrous iron.

On enrichment of serum with ferrous ions as well as ferric a red color appears in the serum. The increase in the intensity of this color on addition of iron gives an approximate idea about the iron-binding capacity of the serum (cf. p. 16). This holds true regardless of the valence of the iron.

Barkan (1933) thought he had been able to show that on addition of iron to serum the added iron is adsorbable on aluminium hydroxide, while the native protein-bound iron is not adsorbed.

He performed only a couple of experiments on serum (most of the experiments on whole blood) and from them he drew the conclusion that it is possible by aluminium hydroxide adsorption to separate the original protein-bound iron from the iron added to the serum. On going through his figures as recorded, however, it will be noticed that in both of the experiments reported he found a greater iron content of the serum after the adsorption than before the enrichment with iron. He ascribed this difference to the small amount of adsorbent employed.

The experiments described below show that iron added to serum over the saturation limit may be adsorbed on aluminium hydroxide, whereas the protein-bound iron (under the saturation limit) is not adsorbed. It is further demonstrated that ferrous ions and ferric ions enter into complex linkage with the serum protein in the same quantity.

Adsorption experiments with Al_2O_3 (Merck's preparation for chromatographic adsorption analysis after Brockmann).

a. Adsorption of serum enriched below the saturation limit.

Serum with an iron content of 30 γ % was enriched with $FeCl_2$ to 176 γ %. The saturation limit, determined after method A, was about 260 γ %. 4 ml. serum was pipetted into each tube, and aluminium oxide was added in amounts varying between 0.2 and 1.5 g. The tubes were shaken for 10 min.; then the adsorbent was separated by centrifuging.

Serum iron determination after the adsorption showed the same value as before the adsorption.

b. Adsorption of serum enriched far above the saturation limit.

Serum with an iron content of 125 γ % was enriched with $FeCl_3$ to 940 γ %. The saturation limit, determined after method A was found to be 230 γ %. The serum was adsorbed with varying amounts of aluminium oxide, and the serum iron content was determined after separation of the adsorbent by centrifuging.

From Table 10 it is evident that with increasing amounts of adsorbent the value for the non-adsorbed iron approaches asymptotically the value for the saturation limit.

c. Adsorption of serum successively enriched above the saturation limit.

Table 10.

Adsorption of loosely bound iron with Al_2O_3 (Brockmann).

Serum ml.	Al_2O_3 g.	γ % iron after adsorption
5	0.5	433
5	1	289
5	2	251
5	3	238

Table 11.

Adsorption experiment (Al_2O_3) with serum enriched successively over the saturation limit.

Serum ml.	Al_2O_3 g.	γ % iron in serum after addition of Fe	γ % iron after adsorption
6	2	(110)	115
6	2	194	201
6	2	277	225
6	2	361	233
6	2	614	240

The initial iron content of the serum was 110 γ %. Then serum was enriched with $FeCl_2$. The saturation limit, determined after method A, was found to be 217 γ %.

From Table 11 it will be noticed that before the saturation limit was reached no iron was adsorbed on the aluminium hydroxide. After the serum was enriched above the saturation limit all further addition of iron was adsorbed almost quantitatively on the aluminium hydroxide.

By measuring pH after adsorption it was found that, through the influence of the aluminium oxide, pH was shifted to about 9 by employment of 2 g. adsorbent. As these experiments primarily were aimed to examine the iron-binding capacity of serum at physiological pH, the adsorption method with aluminium oxide (Brockmann) was not elaborated further. From the experiments recorded above, however, it is evident that with the amount of adsorbent here employed the native protein-linked iron is not adsorbed at pH 9, that iron bound in enriched serum within the saturation limit behaves like the native serum iron as far as

adsorption is concerned, that the iron-binding capacity of serum appears to be about the same at physiological pH as at pH 9, and that ferrous ions as well as ferric ions are bound in a non-adsorbable form up to the saturation limit.

Adsorption experiments with aluminium C γ .

In the above adsorption experiments with Brockmann's aluminium oxide the pH of the media was shifted in alkaline direction. Now aluminium C γ was prepared after Willstätter, Kraut & Erbacher (1925). When this adsorbent was mixed with serum or buffered solutions, the shifting of pH was insignificant. Its adsorbent affinity proved to be high for ferric ions as well as for ferrous ions.

a. Adsorption of ferrous and ferric ions with aluminium C γ (Table 12).

40 ml. phosphate buffer (Sorensen) with pH 7.7 was enriched in one experiment with FeCl₂, in another with FeCl₃, and distributed on two series of tubes. Then different amounts of AlC γ were added. Water was added to make the same volume in all the tubes. The iron concentration of the solutions was determined, after the adsorbent had been separated by centrifuging. The results are given in Table 12.

Table 12.

Adsorption of ferrous and ferric ions with AlC γ at pH 7.

AlC γ ml.	Buffer ml.	H ₂ O ml.	Fe ⁺⁺⁺ γ 0/o free	Fe ⁺⁺ γ 0/o free
0	5	4	640	640
0.2	5	3.8	53	93
0.5	5	3.5	23	87
1.0	5	3	15	15
2.0	5	2	9	6
4.0	5	0	7	15

The influence of the pH of the mixture on the adsorption of ferrous and ferric ions on AlC γ is illustrated by the following experiment (Table 13):

Phosphate buffers (after Sorensen) were enriched with iron to about 700 γ 0/o. Then AlC γ was added and the specimens were shaken repeatedly. After centrifuging for 30 min. the iron content of the solutions was determined.

Table 13.

Adsorption of ferrous and ferric ions with AlC γ at varying pH.

AlC γ ml.	Buffer ml.	pH at adsorption	Fe ⁺⁺⁺ γ % after adsorption	Fe ⁺⁺ γ % after adsorption
0	5		700	700
0.5	5	8.5	14	169
0.5	5	7.2	10	45
0.5	5	6.5	4	12
0.5	5	5.9	6	100

From Table 13 it will be noticed that, within the pH interval here employed, Fe⁺⁺⁺ is adsorbed more readily as Fe⁺⁺. Additional experiments show that Fe⁺⁺ is adsorbed very poorly when pH of the medium is less than 5.5, whereas Fe⁺⁺⁺ is adsorbed very well even with pH as low as 4.

b. Adsorption experiment with AlC γ and serum enriched far above the saturation limit.

100 ml. serum was divided in two equal parts. One portion was enriched with FeCl₂, the other with FeCl₃, to about 1120 γ %. Two series of tests were made, in which the serum was divided in 5 ml. portions, to which AlC γ was added in varying amounts. Water was added to make the same volume in all the tubes. After thorough shaking, the tubes were left standing for 30 min., whereafter the adsorbent was separated by centrifuging, and the amount of serum iron was determined on the solutions. The results are recorded in Table 14. In order to make the results more easily comparable with the initial saturation limit of the serum — which was determined after method C and found to be 290 γ % — the values obtained for the various tubes were converted to values for undiluted serum.

Table 14.

Adsorption of loosely bound iron, ferrous and ferric, with AlC γ .

Serum ml.	AlC γ ml.	H ₂ O ml.	Iron content (γ %) of serum after adsorption	
			Fe ⁺⁺	Fe ⁺⁺⁺
5	0.2	3.8	785	955
5	0.5	3.5	632	860
5	1	3	544	700
5	2	2	452	488
5	3	1	410	386
5	4	0	386	366
5	6	0	368	340

By checking up the pH values it was found that the serum pH prior to the adsorption was 8.5, and after the adsorption 8.4. Thus pH was rather too alkaline for optimal adsorption of the iron. The results show, however, that with increasing amounts of adsorbent the iron concentration of the specimens approaches the saturation limit (290 γ %).

c. Adsorption experiments with $\text{AlC}\gamma$ and serum successively enriched up to and above the saturation limit (Table 15).

In a series of tubes the serum was enriched with increasing amounts of iron. The same amount of $\text{AlC}\gamma$ was added to all the tubes, which were left standing for 90 min. Then the adsorbent was separated by centrifuging, and the iron content of the solution was determined. The iron values obtained were converted to values for undiluted serum. The adsorption took place at pH 7.8. In one series of tubes the serum had been enriched with FeCl_2 , in the other series with FeCl_3 . The saturation limit of the serum, determined after method C, was 290 γ %.

Table 15.

Adsorption experiments ($\text{AlC}\gamma$) with serum enriched gradually over the saturation limit with ferrous and ferric iron.

Serum ml.	$\text{AlC}\gamma$ ml.	Iron content of serum before adsorption approx. γ %	Fe^{++} enrichment. Iron content after adsorption γ %	Fe^{+++} enrichment. Iron content after adsorption γ %
5	4	80	—	—
5	4	180	175	150
5	4	230	200	—
5	4	280	248	240
5	4	380	308	287
5	4	480	316	298
5	4	580	308	294
5	4	680	310	298

In order to demonstrate the great affinity of the iron-binding protein for iron, the last experiment was now modified. Increasing amounts of iron were adsorbed with 4 ml. $\text{AlC}\gamma$, and then 5 ml. serum was added. The mixture of adsorbent and serum was left standing for 90 min. in order to enable the serum to take up iron from $\text{AlC}\gamma$. Then the adsorbent was separated by centrifuging, and the iron content of the serum was determined. The protein

Table 16.
Elution of adsorbed Fe^{++} ions with serum (iron-binding component) from $AlC\gamma$.

Enrichment in both experiments with Fe^{++}	
Fe^{++} added to serum Iron content after adsorption γ %	Fe^{++} added to $AlC\gamma$ Iron content after elution γ %
175	
200	156
248	177
308	206
316	250
308	280
310	305
	299

content of the serum before and after the adsorption was respectively 7.1 % and 6.6 %. The results are given in Table 16.

As the same serum and corresponding amounts of iron were employed here as in the preceding experiment, the results here obtained are directly comparable with the Fe^{++} values in Table 15.

If the serum is enriched above the saturation limit, it is possible to demonstrate that addition of complex binders of iron (pyrophosphate, citrate and tartrate) inhibits the adsorption of the iron added in excess of the saturation limit.

Conclusion.

From the above experiments it is evident that serum is able to bind iron up to a certain concentration in such a form that the iron cannot be adsorbed on aluminium oxide (Brockmann) or on $AlC\gamma$. But if iron is added to serum in a higher concentration, all the iron above this border concentration is adsorbable to the above-mentioned adsorbents. This border concentration agrees quantitatively with the value for the saturation limit, and this affords additional evidence in support of the view that serum contains an iron-binding protein which in native state is more or less saturated with iron.

Furthermore, it can be demonstrated that regardless whether the serum is enriched with ferrous ions or with ferric ions, it takes up the same amount of iron in firm complex binding.

Starkenstein & Weden (1928) have shown that ferrous salts added to serum soon are oxidized. If the serum is enriched with ferrous ions in the presence of hydrosulphite, indeed, this oxidation is prevented. The fact that the iron is taken up by the iron-binding protein in the presence of hydrosulphite thus suggests very strongly that this protein is capable of linkage with ferrous ions. The adsorption experiments reported above, however, indicate that also ferric ions may enter into complex linkage with the iron-binding protein of the serum to the same extent.

Does the Native Serum Iron-protein Complex contain Ferrous or Ferric Iron?

In the literature the view is generally accepted that in native serum the iron is present as ferric iron. This assumption is based on observations reported by Starkenstein & Weden 1928, Barkan 1933 and Tompsett 1934, 1940.

Starkenstein & Weden showed that when ferrous iron is added to whole blood, it is rapidly oxidized to ferric iron. They determined the ferric iron content of the serum by addition of KSCN to a protein-free filtrate that was obtained after precipitation of the serum with hydrochloric acid and trichloroacetic acid. The experiments were carried out with very large additions of iron (iron concentration in serum >1 mg. %). In filtrates from normal sera the color ($\text{Fe}/\text{SCN}/_3$) was found to be so weak that it was difficult to estimate how much was ferric or ferrous iron. As ferrous iron added to whole blood is oxidized to ferric iron, the authors found it justifiable to assume that the native serum iron-protein complex contains ferric iron.

Barkan obtained results which agreed so well with those reported by Starkenstein & Weden that he found it reasonable to subscribe to their view.

Tompsett (1934, 1940) was able to confirm the observation made by Lintzel (1931) that ferric — but not ferrous — iron forms complexes with biological materials. Tompsett states that the iron could be liberated from such complexes with pyrophosphate. (No experiment with native serum is reported, however.)

The iron was also liberated from these complexes by reducing substances (thiolacetic acid, sodium hydrosulphite). On addition of dipyridyl and large amounts of reducing substance to the serum, dipyridyl iron was formed, and this could be extracted with alcohol. No color was obtained without addition of reducing substance. From these findings Tompsett arrived at the following conclusion: »The Fe of the plasma has been found to be wholly in ferric state and as such undissociated». »It seems very probable that at some stage the plasma Fe will at least in part be changed into the ferrous state, *i.e.*, become capable of dissociation and ultrafiltrable.»

Several arguments may be advanced, however, against the idea that the reported experiments prove the native serum iron to be present in ferric form.

The claims made by Starkenstein & Weden and by Barkan are based on the oxidation of ferrous iron added to whole blood. In the native serum iron complex the iron is linked in a way different from that of iron added in excess of the saturation limit. Consequently the valence of firmly bound iron need not necessarily be the same as that of loosely bound iron. The results reported by the cited authors indicate merely that loosely bound iron is found in the ferric form when the serum is enriched by addition of iron to whole blood.

It may be mentioned, moreover, that if freshly withdrawn serum is treated with hydrochloric acid and trichloroacetic acid after Starkenstein's method, and if then potassium thiocyanide is added to the protein-free filtrate, only about one-half of the iron will react under formation of ferrithiocyanide (Table 17). Full development of the color is obtained only when hydrogen peroxide is added to the filtrate. The results indicate rather that in the native serum iron complex the iron occurs partly in oxidized form, partly in reduced form. This is particularly uncertain, however, because the precipitation of protein and liberation of iron in acid milieu may bring about a change in the valence of the exceedingly small amounts of iron here concerned.

Tompsett has not shown that the iron in the serum may be liberated with pyrophosphate. He found that it can be liberated with a reducing substance. But from the findings in Chapter II

Table 17.
*Valency of iron in native serum after
 Starkenstein and Weden.*

Case No.	Serum iron γ 0/0	Ferrous iron 0/0	Ferric iron 0/0
1	108	57	43
2	156	49	51
3	132	69	31
4	187	58	42
5	120	48	52

p. 49 it is plainly evident, however, that at pH 7.2—8 the serum iron complex is not dissociated even in the presence of a reducing substance. When Tompsett was able with a reducing substance in part to transform the iron into a dipyridyl-reacting form, it was because the amounts of reducing substance (hydrosulphite and thiolacetic acid) employed brought about that the serum pH became slightly acid — and then the serum iron complex begins to dissociate (Chapter II p. 51).

From the experimental results given in Table 21 it is evident that when a fresh serum cautiously is acidified in the presence of phenanthroline to pH 5, about 90 % of the iron will react with phenanthroline even without any addition of a reducing substance. This might possibly be taken to indicate that in the serum iron complex the iron occurs in ferrous form. But, as shown by Tompsett, ferric iron is reduced to ferrous iron on cautious addition of acid in the presence of protein.

So it cannot be looked upon as proved that the iron in the serum iron complex is present in ferric form. The experimental findings reported so far may just as well be interpreted to the effect that in the serum iron complex the iron may occur in ferric as well as ferrous form.

Experiments with Dialysis of Iron-enriched Sera.

Intravenous injection of simple iron salts (ferrous or ferric) in excess of the iron-binding capacity of the serum produces a sensation of pressure in the head, irritation of the nasal mucosa,

flushing of the face and nausea. In 10—15 min. after the injection the serum iron value falls to the saturation limit, whereafter it decreases very slowly. Thus the iron exceeding the saturation limit leaves the blood stream rapidly, while diffuse vascular symptoms appear. This indicates that ionized iron introduced in excess of the saturation limit leaves the blood stream by simple dialysis through the capillaries.

Barkan (1927) and Vahlquist (1941) studied the dialyzability of iron in the serum protein-iron complex and found it to be entirely undialyzable at physiological pH. Like Starkenstein & Harvalik (1933), Vahlquist found that even when the serum was enriched with iron up to 1000 γ %, all the iron was still bound in an undialyzable form. These investigators used physiological salt solution for the dialysis. Vahlquist fails to state whether he employed ferrous or ferric iron.

Heubner (1926) and Lintzel (1931) state that ferric — but not ferrous — iron forms non-dialyzable compounds with proteins.

Tompsett (1940) has reported some experiments on dialysis of serum enriched partly with ferrous iron, partly with ferric iron. He found that while »ferrous Fe dialyzed easily, ferric dialyzed hardly at all». In his experiments the dialysis was made against physiological salt solution.

But experiments in vitro with dialysis of serum against saline will not give results that directly reflect the conditions in vivo. Differences in the dialyzability in vivo and in vitro vary with the substances under analysis. The difference between dialysis in vivo and in vitro is due not only to the circumstance that the artificial semipermeable membranes are not of the same quality as the cell membranes but also, to the fact that in dialysis in vivo the media on both sides of the membrane contain protein. This fact has to be taken into consideration in the discussion of the dialyzability of substances forming protein complexes of a low degree of dissociation at physiological pH — and iron just forms such a complex with protein. So the degree of dissociation of this iron-protein complex is bound to have a great influence on the dialyzing rate of the iron ions and on the iron concentration of the media in which an equilibrium is obtained when serum is dialyzed against saline.

Owing to the marked tendency of protein to complex binding of the iron ions, their coefficient of activity in an iron-enriched serum is only small. So when an iron-enriched serum is dialyzed against a medium (*e.g.*, saline) in which the activity coefficient is considerably higher, the dialytic equilibrium is reached already after the passage of an insignificant amount of iron. If, on the other hand, serum is used as external medium, a larger amount of iron will pass through the membrane before a dialytic equilibrium is established, when the same dialytic volume is employed as in dialysis against saline. This is due thus to the circumstance that the protein in the external medium inhibits the activity of the iron ions as compared with the influence of the sodium chloride.

In serum enriched in excess of the saturation limit the loosely bound iron will react with phenanthroline in the presence of hydrosulphite. The loosely bound iron that is obtained by enrichment of serum with ferric or ferrous iron, can also be adsorbed on aluminium C γ (p. 37). These facts indicate that, in contrast to the native serum iron-protein complex, the complexes (in ferric or ferrous form) that iron forms with the serum protein on enrichment over the saturation limit are ionized in part at physiological pH. Thus the loosely bound iron ought to be dialyzable.

In order to investigate the dialyzability of the iron in firmly and in loosely bound iron a number of dialyzing experiments were carried out with employment of serum as external medium — for acceleration of the dialysis.

Iron-enriched serum dialyzed against native serum.

a. Various portions of the same serum were enriched with increasing quantities of ferrous iron (ascorbic acid-reduced FeCl_3).

10 ml. enriched serum was dialyzed in a cellophane tube against 60 ml. native serum for 24 hours, under continuous shaking at a temperature of about 5°. During the dialysis the pH of the external serum varied between 7.7 and 7.5. The iron content of the inner medium was determined before the commencement of the dialysis and after 24 hours. The saturation limit of the inner medium prior to the dialysis was 290 γ %. The serum iron concentration of the outer medium prior to the dialysis was 110 γ %, its saturation limit 260 γ %.

Table 18.

Serum enriched with ferrous iron dialyzed against native serum.

Iron concentration in γ %	
before dialysis	after dialysis
80	86
268	278
345	304
442	317
510	328
680	356

Table 19.

Serum enriched with ferric iron dialyzed against native serum.

Iron concentration in γ %	
before dialysis	after dialysis
101	103
183	183
294	307
480	450
645	546
930	710

The experimental results are recorded in Table 18. During the dialysis the protein concentration of the inner medium rose about 5 %. The iron concentration of the serum after the dialysis has been corrected with regard to the shift in volume — in order more clearly to illustrate at which iron concentration of the serum the iron was non-dialyzable. This applies also to the other dialyzing experiments recorded tabularly.

b. The above experiment was repeated, but this time the serum was enriched with *ferric* iron. The same serum was used as outer medium. In this case the saturation limit of the inner medium was 380 γ %. During the dialysis the pH varied between 7.7 and 7.9. The experimental results are recorded in Table 19.

c. The serum was enriched with ferric iron and dialyzed against physiological salt solution in the presence of pyrophosphate.

Various portions of the same serum were enriched with increasing quantities of ferric iron. To each 10 ml. portion of the serum was added 0.2 ml. sodium pyrophosphate (2.5 %) and a couple of ammonium sulphate crystals, and the serum was dialyzed against physiological salt solution (about 300 ml.) containing 10 ml. phosphate buffer (pH 7.5). The dialysis took place under continuous shaking at a temperature of about 5°. It was discontinued after 20 hours. The saturation limit of the serum prior to the dialysis was 350 γ %. The serum pH at the discontinuance of the dialysis was 7.2. The results obtained are given in Table 20.

Table 20.

*Serum enriched with ferric iron and sodium pyrophosphate,
then dialyzed against physiological NaCl solution.*

Iron concentration in γ ‰	
before dialysis	after dialysis
108	119
205	210
302	290
400	366
594	388
788	476

Conclusion.

In Tables 18, 19 and 20 some typical examples are given of the results obtained in dialyzing experiments with iron-enriched sera.

From these experiments it is plainly evident that when serum is enriched with iron under the saturation limit, all the added iron is bound in non-dialyzable form at physiological or slightly alkaline pH. This holds true whether the serum is enriched with ferrous or ferric iron. Even in the presence of pyrophosphate the iron is non-dialyzable too. This result lends support to the assumption that a compound present in the serum is able to bind the iron in a complex up to the saturation limit, and that this complex is practically not dissociated at physiological pH.

From the experimental results it also is evident that if serum is enriched with ferrous iron over the saturation limit the loosely bound iron is easily dialyzable. If the serum is enriched with ferric iron — instead of ferrous iron — the iron dialyzes considerably more slowly than after enrichment with ferrous iron. But this iron is not entirely non-dialyzable as claimed by Starkenstein & Harvalik (1933) and Vahlquist (1941). On the other hand, the findings reported by Tompsett (1940) are more in agreement with my experiments, when he says: »Whereas ferrous Fe dialyzed easily, ferric dialyzed hardly at all». Tompsett performed his experiments with saline instead of serum as outer medium, and this explains why he arrived at the conclusion »hardly at all». But if pyrophosphate is added to a serum enriched with ferric

iron before the dialysis commences, the iron passes out in the outer medium about just as rapidly as when the serum is enriched with ferrous iron even when the dialysis is carried out against physiological salt solution. This appears to be due to the circumstance that loosely bound iron in ferric form reacts with pyrophosphate, resulting in an easily dialyzable iron complex.

The observations made on the dialyzability of loosely bound iron seemed compatible with the assumption that the toxic reaction appearing on intravenous injection of iron over the saturation limit is due to extravascular (possibly intra-endothelial) occurrence of ionized iron.

Only insignificant amounts of iron pass out into the urine also on intravenous injection of iron above the saturation limit. A contributory cause of this is presumably found in the fact that the activity coefficient for iron ions is higher in the primary urine than in endothelium and extravascular body fluid owing to their protein content.

Stability of the Serum Iron-protein Complex at Varying pH.

The serum iron complex is generally looked upon as being undissociated at neutral reaction. The conditions under which the complex gives off its iron were studied first by Barkan (1927) who found the iron to become ultrafiltrable when serum was acidified with hydrochloric acid to pH ca. 1.3.

Tompsett (1933) studied ferric iron complexes with biological material. He was able to liberate the iron from such a complex by means of large amounts of reducing substances (*e.g.*, thiolacetic acid and sodium hydrosulphite) or with large amounts of pyrophosphate. He performed some experiments with direct addition of dipyriddy to the serum. This gave no coloring of the serum, but »on addition of a reducing substance, *e.g.*, sodium hydrosulphite or thiolacetic acid a red colour appeared. It appears that the iron of serum is in the ferric state.» Tompsett fails to give the serum pH after the addition of the reducing substance, but the reaction must have become slightly acid.

Vahlquist tried to get an idea of the pH range within which the serum iron-protein complex was stable by means of experiments with dialysis of serum against sodium chloride solutions at varying pH. His results showed that serum iron is non-dialyzable — not only at neutral reaction, but also within a relatively wide range of pH: from 10 to 4.5. From his findings, Vahlquist arrived at the conclusion: »dass das Eisen nicht nur bei der Eigenreaktion des Serums, sondern auch innerhalb einer ziemlich breiten Zone zu beiden Seiten derselben, vollständig komplexgebunden ist».

Writer's Experiments.

Like iron added to serum under the saturation limit, the native serum iron will not at physiological pH react with dipyridyl or phenanthroline even though a reducing substance is present. This implies that at physiological pH practically no ferrous ions are found in the serum. A series of experiments was then carried out in order to settle at which pH the serum iron-protein complex commences to give off iron ions.

Determinations were made of firmly bound non-phenanthroline reacting iron in serum at varying pH. In Fig. 5 the results from a couple of typical experiments of this kind are presented graphically.

Serum was enriched with iron to about two hundred gamma per cent over the saturation limit, and then divided into portions of 10 ml. By means of ammonia and hydrochloric acid the pH was adjusted in the various portions to different values, and then 10 mg. sodium hydrosulphite and phenanthroline were added. Then pH was determined again. One hour later the amount of acid-soluble, non-phenanthroline-reacting, iron in the various portions was determined after the usual method for serum iron determination.

It is evident that the value for non-phenanthroline-reacting iron in serum is constant within the pH interval of 8—7.2. When the determination is made on a more alkaline serum ($\text{pH} > 8$), higher values are obtained. The reason for this has not been investigated.

It will further be noticed that on the acid side of pH 7.2 the iron is dissociated rapidly from the iron-protein complex in the presence of phenanthroline and hydrosulphite. For its reaction

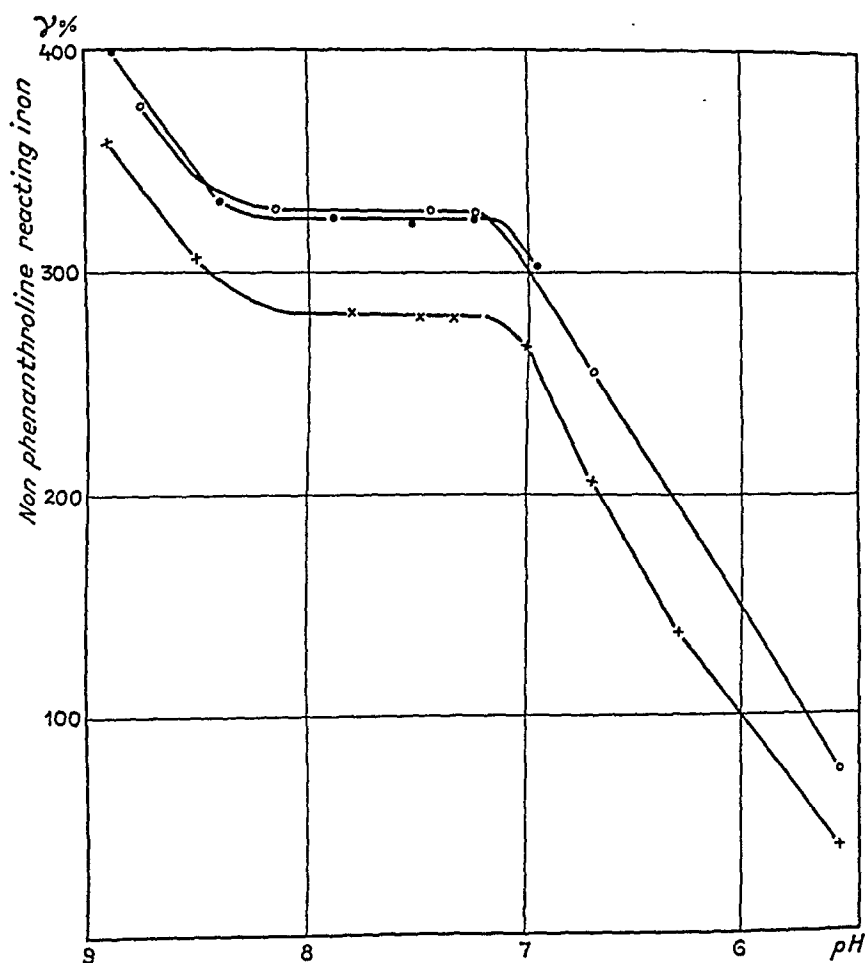


Fig. 5. Iron-binding capacity of serum at different pH values in the presence of phenanthroline and sodium hydrosulphite.

with phenanthroline the iron has to be ionized. Thus, these findings imply that the iron-protein complex begins to undergo dissociation immediately on the acid side of the normal blood pH.

The experiments reported above were carried out with sera that were enriched above the saturation limit with ferrous or ferric iron. Another series of experiments was performed in order to see how the iron would behave in native sera and in sera enriched below the saturation limit when they were acidified to the pH interval of 7—5 in the presence of hydrosulphite and phenanthroline. The findings thus obtained are presented graphically in Fig. 6.

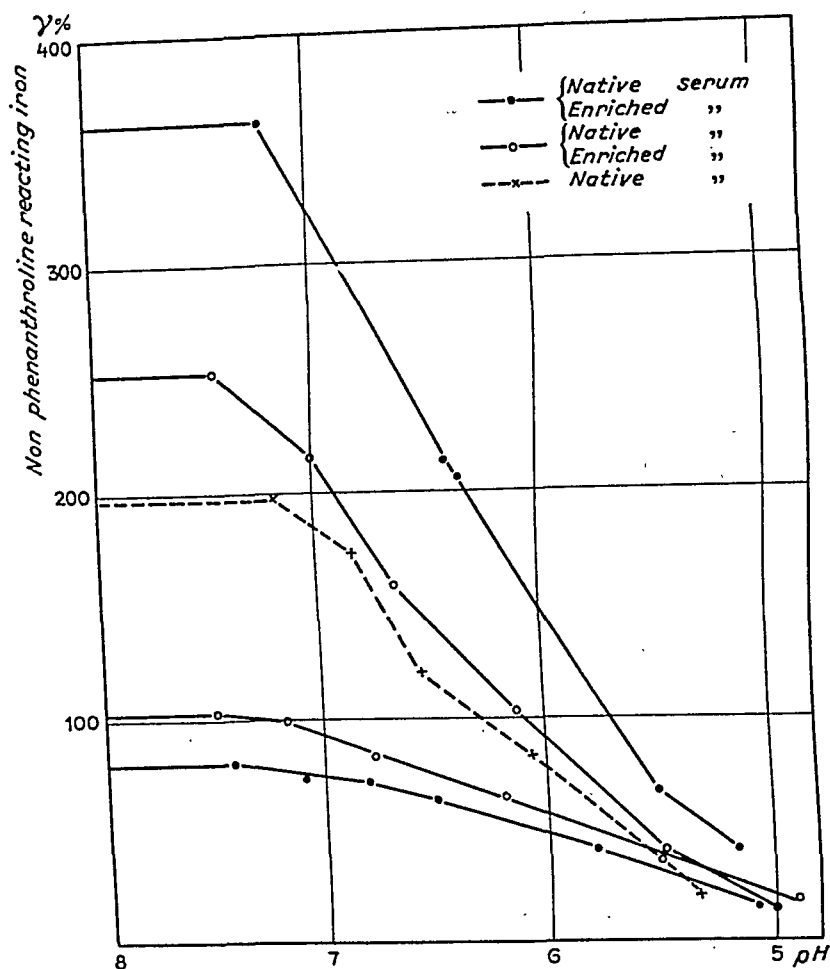


Fig. 6. Non-phenanthroline-reacting iron in serum (native and enriched under the saturation limit) at different pH values.

From Fig. 6 it will be noticed that the type of liberation of iron from the serum iron-protein complex obtained here was the same whether the experiment was performed on a native serum or on a serum enriched with iron below the saturation limit. This corroborates the assumption that iron added to serum under the saturation limit is bound in the same manner as the iron in the native serum iron-protein complex. As phenanthroline is a good complex binder of iron within the pH range of 1.5—9, these findings indicate that the serum iron-protein complex is practically entirely non-dissociated on the alkaline side of pH 7.2 and that it begins to undergo dissociation on the acid side. At pH 5 prac-

tically all the iron in the serum iron-protein complex is liberated in the presence of phenanthroline and hydrosulphite. From these experiments, however, no definite conclusions may be drawn other than that the native serum iron-protein complex begins to undergo dissociation on acidification of the serum to pH values that lie immediately on the acid side of the physiological pH range.

In his investigations into the conditions for dissociation of the serum iron-protein complex, Vahlquist (1941) thought that he was able to show that dissociation of the complex began first on the acid side of pH 4.5 — which is difficult to bring in keeping with the experimental results recorded above. Still there is this difference in the technique that Vahlquist had no reducing substance present in his experiments. Generally, although on a very weak experimental basis, the iron in the serum iron-protein complex is taken to be trivalent (*e.g.*, Barkan, Starkenstein, Tompsett). In the experiments here described sodium hydrosulphite was added to the analytical specimens. This may perhaps imply that the iron-protein complex is reduced and that the complex binding between ferrous iron and protein is dissociated more readily than is the complex with ferric iron and this in turn might explain the difference in the experimental results obtained. If the iron were bound in ferric form and its valence did not change on acidification of the medium, then no phenanthroline-reacting iron should appear in the serum at pH 4.5 if the dialyzing results reported by Vahlquist were due to the dissociation of the complex. In order to see if this might prove true, *some sera were acidified to pH ca. 5 in the presence of phenanthroline but without addition of any reducing substance.* The results are recorded in part — in some other connection — in Table 21. In order to reduce the methodical error, most of the experiments were carried out on sera with high values for serum iron. *On acidification of fresh sera to about pH 5 in presence of phenanthroline it is generally found that between 80 and 90 % of the serum iron is conveyed into phenanthroline iron.*

The same result is obtained whether the high serum iron is brought about through administration of iron to the experimental subjects prior to the withdrawal of the blood, or whether the serum is obtained from patients with high serum iron (patients

suffering from acute hepatitis or hemolytic jaundice). The dissociation rate of the serum iron complex on acidification of the serum is practically the same whether the experiment is performed with or without addition of hydrosulphite. So these experiments show that either is the iron in the serum iron complex natively made up mostly of ferrous Fe, or some normal components of the serum exert a reducing action when the serum is acidified, so that ferric iron in the complex is transformed to ferrous iron, thus facilitating the dissociation of the complex. A third alternative is that the ferric complex is dissociated already at pH ca. 5 and that the free iron ions secondarily are reduced to the phenanthroline-reacting form. In itself, phenanthroline has no reducing effect on ionized ferric iron.

Still none of the explanations mentioned here appear adequately to explain the discrepancy between the experimental results obtained by Vahlquist and the writer's findings.

Is the Reaction — Iron-binding Component + Iron \rightleftharpoons \rightleftharpoons „Serum Iron“ — Reversible?

In the preceding section it was shown that iron is liberated from the serum iron-protein complex if the serum is acidified to pH ca. 5 after phenanthroline and hydrosulphite had been added. In this section an account will be given of some experiments carried out to see whether the iron may be liberated from the serum iron-protein complex without the iron-binding component of serum losing its iron-binding capacity.

In order to be able to estimate the iron-binding capacity of the serum after the iron has been liberated from the serum iron-protein complex, the phenanthroline and hydrosulphite will first have to be eliminated from the serum, as otherwise they will prevent the iron-binding component from reacting with the iron added to the serum in determination of the saturation limit. The experiments were carried out in the following analytical stages:

- 1) *The values for serum iron and saturation limit were determined on fresh serum.*

These experiments are carried out with employment of sera which in native state showed a high serum iron value (sera from normal subjects who had been given iron, and sera from patients suffering from acute hepatitis or from hemolytic jaundice).

The results are recorded in Table 21, column 1.

2) *The serum was acidified in the presence of phenanthroline to pH ca. 5, so that the serum iron reacted with the phenanthroline.*

20 ml. serum was acidified with 0.5 N HCl to pH ca. 5 after some phenanthroline crystals had been added. After standing for 1 hour a serum iron determination was made on a part of the specimen in the usual way.

The results are recorded in Table 21, column 2, from which it is evident that the serum iron concentration has fallen to about one-tenth of its initial value, i.e., that about 90 % of the serum iron has been converted to phenanthroline iron.

3) *The phenanthroline iron formed and the excess of phenanthroline were eliminated from the serum.*

The phenanthroline and phenanthroline iron may be eliminated from the serum through dialysis, but this takes a couple of days to be done efficiently. Besides, prolonged dialysis implies a great risk of bacterial growth in the serum. Two other methods may be employed:

A. One method is based on the fact that phenanthroline and phenanthroline iron can be removed from the serum by absorption of activated charcoal (see p. 17).

Under mechanical stirring, the phenanthroline-treated serum is neutralized to pH ca. 7.5 with ammonia (concentrated ammonia diluted 1:5). Charcoal is added and the mixture is shaken for 5 min., whereafter the charcoal is separated by centrifuging. Now the serum is practically free from phenanthroline as well as phenanthroline iron.

B. The other method is based on the fact that phenanthroline as well as phenanthroline iron are soluble in ethanol.

1 volume phenanthroline-treated serum (pH ca. 5, temperature ca. 5°) is cautiously added dropwise under stirring to 10 volumes alcohol (concentration 99.5 %, temperature -15°). During the following half hour the precipitate is stirred a few times, and throughout this period the specimen is left standing at -15°. Then it is centrifuged, in cold centrifuge tubes, for about 2 min. at a rate of 3500 revolutions per min. The alcohol is poured out, and new cold alcohol is added. The precipitate is stirred up a few times, still in

Table 21.

Influence of liberation of iron from the iron protein complex upon the saturation limit. Sera Nos. 2 and 5 originate from patients with hemolytic icterus; No 4 from acute hepatitis. The other sera from normal subjects given 2—8 mg. Fe⁺⁺ intravenously.

Case No.	1		2	3	4		
	Serum (native)		Serum + phenanthroline acidified during 1 hour to pH	Serum iron after 1 hour γ 0/0	Elimination of phenanthroline and iron with	Serum after neutralization and extraction of phenanthroline and phenanthroline iron	
	Serum iron γ 0/0	Saturation limit γ 0/0					
1	220	256	5.1	14	Charcoal	27	210
2	240	280	5.0	18		124	250
3	291	290	4.8	18		132	282
4	201	304	4.8	25		75	300
5	169	321	5.2	48	ethanol ether	52	290
6	168	252	4.9	20		22	238
7	308	356	4.8	25		21	316
8	173	270	4.9	19		20	258
9	218	316	5.2	72		70	296

thinks that the values obtained with the method employed by Barkan for determination of easily split-off iron hardly may be accepted as an expression for any well-defined iron fraction which normally would be present in the blood cells. From their comprehensive clinical studies on variations of easily split-off iron in various pathological conditions Moore *et al.* (1939) arrived at the result that »the physiological importance of this blood iron fraction has fairly definitely been divorced from the function of iron transportation». Heilmeyer (1937), Moore, Minnich & Welch (1939), Wenndt (1940), Miller & Hahn (1940) and Legge & Lemberg (1941) hold that the easily split-off iron in the blood cells at least to a large extent is an artefact.

Studies have also been reported on the possible passage of iron from the serum into the blood cells. On addition of iron to whole blood Barkan (1933) recovered the added iron quantitatively from the plasma. Moore, Doan & Arrowsmith (1937) found that on marked increase in serum iron through administration there was no change in the value for easily split-off iron in the blood cells.

Hahn, Bale, Ross, Hettig & Whipple (1940) mixed serum containing radioactive iron (from a dog after injection of such iron), with blood cells from other dogs, and found no demonstrable passage of the radioactive iron into the blood cells.

Waldenström (1944) has reported some experiments *in vitro* with addition of iron to whole blood from normal subjects. This addition was made in two experiments so that the calculated increase in serum iron would be respectively 155 and 142 γ % if the iron was distributed only on the serum. In the first experiment the initial serum value was 112 γ %; in the other experiment it is not recorded. Even though in one experiment the plasma was not separated by centrifuging till the following day, practically all the iron added was found again in the plasma. In iron-enriched whole blood from a pernicious-anemic patient the added iron was likewise found quantitatively in the plasma.

Thus the results obtained by the investigators cited above indicate rather that under physiological conditions no passage of iron takes place from the serum to the blood cells or vice versa.

Waldenström's experiments with enrichment of blood from normal subjects as well as the above experiments performed by

Table 22.

Iron enrichment of whole blood in vitro.

Calculated serum iron after enrichment if all the iron is taken up in serum $\gamma^{0.0}$	Serum iron obtained $\gamma^{0.0}$
—	98
186	195
239	234
284	285
337	347
400	386
503	484
648	630
—	67
231	220
395	389
559	520
887	870
1707	1640

Hahn and collaborators with radioactive iron represent investigations on the ability of the blood cells to take up iron from serum that was enriched below the saturation limit. In order to ascertain whether any iron diffuses into the blood cells when the serum is enriched above the saturation limit, the following experiment was performed.

Whole blood, on which the hematocrite value, initial serum iron and saturation limit had been determined, was enriched in a series of tubes with increasing amounts of iron. The blood was left standing for 1 hour and then the blood cells were separated by centrifuging. Iron determination was performed on the plasma.

Table 22 shows the serum iron values obtained as well as the values that were to be expected after the enrichment with iron if all the added iron was distributed on the plasma alone.

From Table 22 it will be noticed that the results here obtained are quite in keeping with those reported by previous investigators, which all indicate that, like other cations, ferrous and ferric ions do not penetrate the corpuscles — at least not in the direction from plasma into corpuscles. This appears to hold true when the addition of iron is so small that the iron concentration of the

plasma does not exceed the saturation limit as well as when the iron concentration of the plasma is increased considerably above the saturation limit.

But this does not settle conclusively the question whether the blood cells contain any protein-linked iron fraction of the same type as found in the plasma. The method for determination of serum iron is too unspecific to afford an answer on this question by a »serum iron determination» on the blood cells. Such a test will merely give an expression for the amount of easily split-off iron in the blood cells (see above), which Barkan has designated as fraction E. If the blood cells are treated with carbon dioxide prior to the determination, a lower value is obtained (Barkan's fraction E_1). As emphasized above, these fractions (E and E_1) are taken at least to a large extent to be methodical artefacts.

The same protein-linked copper fraction as is found in plasma (hemocuprein) has also been obtained in pure form from erythrocytes (Mann & Keilin 1938). Analogously it would not be improbable that the same iron-containing protein fraction as is found in the plasma might be found also in the erythrocytes. As it had not been possible yet to decide whether this be the case, the writer has investigated the latent iron-binding capacity of whole blood as compared to that of plasma.

The hematocrite value was determined on heparin blood. The serum iron and the saturation limit were determined on plasma separated by centrifuging. The whole blood was hemolyzed by repeated freezing and thawing.

The completely hemolyzed blood was distributed on three series of tubes (5 ml. in each tube). In one tube it was enriched with Fe^{++} , so that the iron concentration was increased by 200 γ %. In another tube a corresponding amount of Fe^{+++} was added to the blood. For removal of the loosely bound iron the specimens were shaken with $AlC\gamma$ for one hour and then the adsorbent was separated by centrifuging. The clear supernatant fluid was then shaken for 10 min. in an atmosphere of carbon monoxide, whereafter the serum iron was determined.

As pointed out before (p. 40), loosely bound iron can be adsorbed on $AlC\gamma$, while firmly bound iron and iron-binding component in serum are not adsorbed. Hemoglobin is partly adsorbed

Table 23.

Determination of iron-binding capacity of whole blood and plasma.

Case No.	Serum iron γ $^0/0$	Saturation limit γ $^0/0$	Hematocrite value	Easily split-off iron in whole blood (non-adsorbable on AlC γ) before and after addition of iron to whole blood. γ $^0/0$		Capacity of whole blood to bind further iron γ $^0/0$	Capacity of plasma in whole blood to bind iron. (Calculated from the values for saturation limit, serum iron and hematocrite. γ $^0/0$)
1.	132	328	43	0 Fe	515		
				+ Fe $^{++}$	655	140	112
				+ Fe $^{+++}$	680	165	112
2.	86	324	46	0 Fe	442		
				+ Fe $^{++}$	572	130	127
				+ Fe $^{+++}$	546	104	127
3.	146	340	48	0 Fe	204		
				+ Fe $^{++}$	286	82	101
				+ Fe $^{+++}$	296	92	101

on AlC γ . The difference between the iron values obtained in the iron-enriched and the non-enriched specimens after adsorption on AlC γ will thus be an expression for the latent iron-binding capacity of the hemolyzed whole blood. By means of the values for serum iron, saturation limit of the plasma and hematocrite it is then calculated how great the increase in non-adsorbable iron should be if the iron-binding component were found in the plasma. The results are recorded in Table 23, which shows the values obtained for the latent iron-binding capacity of whole blood as compared to the values that might be expected if the iron-binding component were found in the plasma alone.

The methodical error in these determinations is considerably greater than that in ordinary determination of serum iron or saturation limit, because the precipitation of protein by acidification in the presence of hemoglobin is made up of large floccules. The difficulty in obtaining clear filtrate makes the filtration time variable. In this way, the acid will have a chance of acting for some varying length of time on the solution which is markedly hemoglobin-containing also after the adsorption. The longer the filtration time, the higher values will be obtained for iron in the

filtrate, as more hemoglobin will then be broken down. When this is taken into consideration in judging of the results obtained (Table 23), these seem indisputably to indicate that the iron-free iron-binding component is present in the plasma in a much higher concentration than in the red blood cells, that is: the red blood cells are practically devoid of any latent iron-binding capacity.

CLINICAL INVESTIGATIONS.

Chapter IV.

Serum Iron and Saturation Limit of Serum in Normal Adults.

Normal Variations.

Material. These determinations were performed in subjects who were feeling perfectly well and had not gone through any kind of infectious disease within the last week. The majority of these experimental subjects was made up of persons who had volunteered as donors for blood transfusions without having given any blood previously. The remaining specimens of blood came from medical students and nurses.

The material comprises altogether 100 normal subjects. The analytical results are recorded together in Table 25. No selection of the material has been made. The withdrawal of the blood was not limited to any definite part of the day.

The average values obtained for serum iron in men as well as women show satisfactory agreement with the findings reported by previous investigators. Some of the more recent results obtained from large materials are cited in Table 24.

Different methods have been employed by the authors cited (methods based on the rhodanide reaction as well as on the phenanthroline reaction), but the results obtained are quite in harmony, and the sex difference is seen to be more or less pronounced in all the materials. To the sex difference in serum iron no corresponding sex difference is found for the saturation limit. The mean value for saturation limit in men and women is 315 γ %.

Table 24.

Normal values for serum iron in healthy persons.

Investigator	Year	No. of cases	Serum iron γ %
Skouge	1939	50 M	118 (79—158)
		50 F	104 (66—164)
Vahlquist	1941	50 M	142 (68—263)
		50 F	123 (53—210)
Br�chner-Mortensen ...	1943	50 M	128 (78—194)
		50 F	118 (79—191)
Hemmeler	1946	100 M	132
		100 F	103
Laurell.....	1947	61 M	124 (70—214)
		39 F	108 (57—196)

With a view to the systematic methodical error, the real mean value for the present material is estimated as 310 γ %. In their previous studies, Holmberg & Laurell found a mean value of 312 γ % for 10 normal subjects. Schade & Caroline (1946) estimate the iron-binding protein content of serum to be 260 γ % in a normal man weighing 70 kg.

As there appears to be no sex difference in this respect in the present material the total number of subjects have been treated statistically under one, with this result: $M=315 \pm 3.3$ γ %.

No simple correlation is found between serum iron and the iron-binding protein content. Considering the individual cases, the iron-binding protein content is seen not to vary parallelly with the serum iron. The former is decisive of the serum iron concentration insofar as it represents the upper variation limit for serum iron in the individual case. The actual serum iron level is a complex function of the more or less known variables of the iron metabolism where the iron-binding protein merely constitutes one of these variables.

Table 26 shows the age distribution of the normal material. A preponderant part of this material falls between 15 and 40 years. In this material no difference is observed between the values for the saturation limit obtained for the different age groups.

It may be pointed out, however, that at parturition the newborn shows a saturation limit which is decidedly lower than that of

Table 25 a. *Serum iron and saturation limit in 61 men.*

Age	Serum iron γ %	Satura- tion limit γ %	Manifest Latent iron- binding capacity	Age	Serum iron γ %	Satura- tion limit γ %	Manifest Latent iron- binding capacity
22	138	406	0.51	37	150	312	0.93
31	93	400	0.30	25	195	310	1.69
42	149	365	0.68	50	98	310	0.46
27	133	363	0.57	20	163	308	1.12
18	76	362	0.26	41	89	307	0.41
21	162	361	0.81	28	193	307	1.69
26	117	356	0.49	26	209	306	2.15
22	192	352	1.20	42	70	305	0.30
24	120	350	0.52	18	80	305	0.35
16	142	349	0.68	31	86	304	0.39
38	149	346	0.76	26	107	299	0.56
23	86	344	0.33	22	116	297	0.64
22	182	344	1.12	26	160	296	1.18
25	79	343	0.29	20	103	295	0.54
38	105	339	0.45	23	101	293	0.53
19	201	337	1.48	24	126	293	0.75
26	98	332	0.42	20	88	293	0.43
21	193	331	1.40	33	107	290	0.59
29	144	330	0.77	27	71	284	0.33
16	100	330	0.44	24	89	283	0.46
32	214	328	1.88	27	171	277	1.61
48	126	328	0.62	25	109	275	0.66
26	89	326	0.37	23	105	275	0.62
19	80	325	0.33	24	106	275	0.63
30	122	320	0.62	27	94	269	0.54
29	101	319	0.46	31	80	261	0.44
22	115	319	0.56	24	96	260	0.59
18	90	316	0.40	26	109	260	0.72
20	138	316	0.77	26	96	255	0.62
20	107	313	0.52	21	206	254	4.29
22	122	313	0.64	Mean value	124	315	0.77

Table 25 b. *Serum iron and saturation limit in 39 women.*

39	57	395	0.17	24	87	310	0.39
19	96	384	0.33	18	91	308	0.42
26	112	370	0.43	17	138	307	0.82
33	82	369	0.29	22	132	306	0.76
22	111	367	0.43	35	100	300	0.50
41	154	356	0.76	61	107	300	0.55
19	114	342	0.50	32	196	297	1.94
22	117	340	0.52	46	96	297	0.48
20	80	339	0.31	24	78	295	0.36
33	103	337	0.44	40	101	294	0.52
19	130	333	0.64	35	155	292	1.13
35	100	324	0.43	22	84	291	0.41
58	73	324	0.29	40	110	291	0.60
22	111	322	0.53	23	64	280	0.30
40	98	322	0.44	56	101	280	0.56
35	172	317	1.19	39	129	278	0.87
48	108	315	0.52	34	136	273	0.99
60	118	314	0.60	49	65	265	0.33
44	75	312	0.35	19	57	245	0.30
20	167	311	1.16	Mean value	108	315	0.58

Table 26.
Age distribution of the normal material.

Saturation limit γ %	Age						
	15—19	20—24	25—29	30—39	40—49	50—59	60—69
241—260	1	2	2				
261—280		3	3	3	1	1	
281—300		8	3	4	3		1
301—320	4	8	4	4	4	1	1
321—340	4	4	3	4	2	1	
341—360	2	4	2	1	1		
361—380	1	2	2	1	1		
381—400	1			2			
401—420		1					

the adult organism, and this difference is statistically established. The mean value for 25 newborn children was $226 \pm 10 \gamma$ %, with a serum iron concentration of 147γ % (tab. 31). How rapidly the normal adult value for iron-binding protein is attained is still to be investigated.

Table 25 gives also the quotient $\frac{\text{manifest}}{\text{latent}}$ iron-binding capacity. For it has been found (Chapter VIII) that the value for this quotient gives some information about the direction of the iron transport at the time of the analysis in the individual case.

Relation between Abrupt Changes in Serum Iron and the Saturation Limit.

In a number of cases iron was given by mouth in order to ascertain how the saturation limit was influenced by the absorption of iron. For oral administration of a fairly large amount of iron to normal subjects gives a rapid rise in the serum iron followed by a slower fall.

The serum iron concentration and saturation limit were determined on 10 experimental subjects before and after ingestion of iron (ferrous tartrate, 0.55 g. Fe^{++}). The second determination was made after 4 hours because previous investigations had shown that the highest values for serum iron then are obtained after

Table 27.

Iron tolerance test with 0.55 g. Fe^{++} by mouth. Samples of blood taken before and 4 hours after intake of iron.

Name	Sex	Age	Serum iron (γ %) after		Saturation limit (γ %) after	
			0 hours	4 hours	0 hours	4 hours
S.I.	F	23	161	320	339	334
S.S.	F	26	134	333	328	315
L.S.	M	34	162	192	274	266
G.T.	M	23	142	192	300	288
A.C.	M	25	118	340	336	338
K.J.	M	25	138	175	316	307
R.J.	M	25	126	193	303	307
F.L.	M	27	210	239	263	267
C.L.	M	27	149	262	280	278
G.P.	M	26	128	186	370	365
Mean value:			147	243	311	307

Table 28.

Iron tolerance test with 0.55 g. Fe^{++} , given by mouth. Samples of blood taken after 4 and 24 hours.

Name	Sex	Age	Serum iron (γ %) after		Saturation limit (γ %) after	
			4 hours	24 hours	4 hours	24 hours
S.G.	F	24	287	86	284	284
H.T.	M	22	154	154	332	330
F.L.	F	37	227	134	334	322
H.B.	M	23	180	154	366	364
G.L.	F	32	289	109	324	326
F.A.	F	35	288	184	320	282
S.C.	F	32	331	78	326	318
S.B.	F	30	243	121	269	264
F.M.	F	23	304	81	315	307
F.K.	F	26	173	113	292	293
Mean value:			248	121	316	309

2—4 hours. Another series of tolerance tests were performed in which the first sample of blood was withdrawn 4 hours after the injection of iron, the other sample after 24 hours, in order to ascertain how the saturation limit behaves when the serum iron falls from the high 4-hour value to normal level (in oral iron tolerance tests the serum iron values usually fall to the initial

values in 12—24 hours). The experimental subjects were all feeling perfectly well. The experimental results are recorded in Tables 27 and 28.

Discussion of the experimental results.

During the period from 0 to 4 hours (Table 27) the serum iron concentration rose from an average of 147 γ % to 243 γ %. During the same period there was practically no change in the saturation limit. In no instance did the variations in the value for the saturation limit exceed the methodical error. Serum iron rose in all the experimental subjects, but this rise was liable to wide individual variation (extreme values: 29 and 222 γ %). In the literature on iron tolerance tests (see, among others, Vahlquist 1944) it is generally stated indeed that the same dose of iron gives an individually most variable rise in serum iron.

The rise in serum iron without any change in the saturation limit suggests very strongly that the iron leaves the intestinal mucosa in ionized form, for, if the mucosa furnished the iron to the blood stream as an iron-protein complex, the saturation limit would have been raised.

Table 28 gives the values for serum iron and saturation limit 4 and 24 hours after the ingestion of iron. Here the serum iron is seen to fall from an average of 248 γ % (after 4 hours) to 121 γ % (after 24 hours). In spite of this fall in serum iron, the saturation limit keeps practically at the same level. Through the extensive American experiments with radioactive iron it has been established that the iron assimilated in tolerance tests partly is stored in the iron depots, partly utilized for synthesis of hemoglobin. As serum iron falls during the period of 4—24 hours after the intake, a part of the iron thus is stored in the depots, and a part is used for synthesis of hemoglobin. Whether the iron is used to synthesize hemoglobin or transferred to storage iron, it leaves the blood stream.

Now the question is: Is the iron liberated from the serum iron protein complex?

a) Without decomposition of the high-molecular iron-binding component or

b) under simultaneous decomposition of the iron-binding component.

The first alternative (a) will bring about:

1) that, after the iron is liberated, the iron-binding component can again circulate in the blood stream and serve as transport for a new iron atom, and

2) that the building rate for the iron-binding component need not necessarily be particularly high.

The second alternative (b) implies:

1) that the building rate for the iron-binding component must be very high, and

2) that the organism must be able quickly to compensate the falls in the iron-binding component content of the serum. If the latter condition is not met the rapid falls in serum iron will make shifts in the saturation limit.

From the experiments reported it is evident that the saturation limit does not vary in spite of the wide variations in the serum iron concentration. This makes first alternative (a) more probably than the second (b). From a chemical point of view the first alternative (a) is not unreasonable, as experiments in vitro (Chapter II, p. 56) show that the iron-binding component may combine with iron in a reversible linkage.

The hypothesis here advanced will be dealt with more thoroughly in Chapter VIII, after an account has been given of variations in the saturation limit in various pathological conditions.

In order to get an idea about how great a part of the latent iron-binding capacity is utilized in the absorption of iron in men or women when the serum iron attains its highest value, the two materials in Tables 27 and 28 are added together with differentiation of the sexes. The 24-hour value is taken to signify the normal serum level. The outcome is given in Table 29.

From Table 29 it is evident that the normal level of serum iron is somewhat higher in men than in women — and generally the same is found in all fairly large materials concerning variations in serum iron. The saturation limit is practically the same in men and women. From Table 29 it is obvious, however, that a greater part of the latent iron-binding capacity of serum is utilized for

Table 29.

Comparison between latent iron-binding capacity of serum in men and women after oral administration of iron.

Sex	Number	Serum iron (γ ‰)		Saturation limit (γ ‰)	Latent iron-binding capacity (γ ‰)	
		At 0 and 24 hours	after 4 hours		at 0 and 24 hours	after 4 hours
Men	10	148	211	313	165	102
Women	10	120	280	309	189	29

the iron transport in women (about 85 ‰) than in men (40 ‰). The explanation of this may be either that women absorb iron more readily than men when large amounts of iron are ingested, or that the iron is eliminated more rapidly from the blood stream in men than in women.

It should be possible to ascertain which of these explanations may be correct — by intravenous as well as peroral administration of iron to subjects of either sex. The rate at which the serum iron falls after intravenous administration gives an expression for the rapidity of its elimination.

In 10 of the experimental subjects the serum iron rose in 4 hours to a value that practically was equal to the saturation limit. In the remaining subjects the values obtained were not so high. The question then arises whether the blood never is able on its passage through the intestinal mucosa to absorb an amount of iron that exceeds the saturation limit. That this actually may take place is by no means excluded even though it does not manifest itself in a rise in serum iron above the saturation limit. For any possible excess of iron will leave the blood stream very rapidly. Ingestion of iron in large doses (up to 1 g.) is often followed by nausea and diarrhea, the explanation of which may very well be that the concentration of ionized iron becomes so high that the iron-binding capacity of the mucosa and serum is overtaxed, resulting in an abnormally high concentration of free iron ions in the intestinal wall, so that the mucosal cells become strongly irritated.

In this connection it is to be pointed out that a considerable rise in serum iron after ingestion of a large dose of iron by no

means signifies that the subject under physiological conditions is able to utilize the iron in his food. For the peroral iron tolerance test is carried out with such a large amount of iron that the mechanism involved in the passage of the iron through the intestinal mucosa need not at all be the normal one. It is quite conceivable that the normal mucosal blockade for iron is broken partially by the high concentration of ionized iron resulting in the digestive tract after fasting ingestion of a large dose of iron.

Chapter V.

Variation in Serum Iron and Saturation Limit during Normal Pregnancy.

Serum iron and saturation limit in women during normal pregnancy.

In the course of pregnancy the mother normally loses about 350 mg. iron to the fetus (McCance & Widdowsson 1937). Thus the gestation implies an increase in the iron requirement. In order to meet the iron requirement of the fetus the pregnant woman must increase her absorption of iron or adjust her intermediary iron metabolism so that the storage iron is mobilized.

In order to elucidate the iron absorption during pregnancy, Raener (1942) performed peroral iron tolerance tests on a number of pregnant women, examining the resulting rise in serum iron. On administration of 225 mg. ferrous iron the serum iron concentration in non-pregnant women increased on an average with 145 γ %, while in pregnancy the average rise was 229 γ %. As the elimination rate of iron from serum ought to be higher in pregnant than in non-pregnant women, his findings suggest very strongly that women absorb iron more readily during pregnancy. Guggisberg (1941), who also made peroral iron tolerance tests on pregnant women has presented some curves for the resulting increases in serum iron. In one of his cases the serum iron rose in 4 hours after the intake of iron to a level over 400 γ %, which may be looked upon as a supernormal increase.

The iron absorption during pregnancy has been investigated also by means of radioactive iron (Balfour, Hahn, Bale, Pommerenke and Whipple 1942). From their findings these authors concluded that the pregnant woman at least in the late months

of pregnancy does show increased intake of radio iron, although there are great variations and a few cases of normal intake». »The pregnant woman as a rule shows 2—10 times the normal absorption of radio iron.» Hahn (1947) reports unpublished investigations by the Vanderbilt Cooperative Study of Maternal and Infant Nutrition, in which the iron absorption was found to be increased in the latter part of pregnancy.

Variations of the serum iron concentration in various months of pregnancy have been examined by Sundelin (1942) on 130 women. During the first five months of pregnancy he found a normal serum iron value (118 γ %). In the sixth month the value began to fall, and the lowest value was obtained in the 8' and 9' month (48 γ %). In the 10' month the values increased again to an average of 80 γ %. Similar studies were carried out by Raener (1942), who in his material found the same tendency to variation of serum iron, though less pronounced. Albers (1941) claims that during pregnancy the serum iron values are constantly increased, but his average value is 120 γ %, which corresponds to the normal value found by most other investigators for non-pregnant women, and his conclusions have no statistical foundation. Besides, his analyses cover only the first and last months of pregnancy.

Writer's Findings.

Table 30 and Fig. 7 give the results of serum iron and saturation limit determinations on 51 women in various months of pregnancy. Fig. 7 also presents the results recorded in Tables 31 and 32, showing the values obtained for serum iron and saturation limit at parturition (25 cases) and in the first days post partum (17 cases). In this material too we meet with the tendency pointed out by Sundelin to variation of serum iron in the various months of pregnancy.

During the first months of pregnancy the values for the saturation limit fall within the limits of normal variation, but in the 6' month they show a distinct tendency to increase, reaching maximum in the 9' month, about 200 γ % over the normal level. The average value at the parturition was 446 γ %. Largely, then, the variations of the serum iron concentration and of the saturation

Table 30.

Serum iron and saturation limit in various months of pregnancy.

Name	Age	Number of parturitions	Last normal menses on	Blood sample taken on	Serum iron γ %	Saturation limit γ %
A.A.	30	0	10/11	28/12	94	320
A.P.	39	1	2/11	2/1	171	377
E.G.	32	0	28/11	11/2	74	340
E.N.	22	2	31/10	9/1	104	290
S.N.	27	3	1/11	21/1	111	369
P.P.	35	2	18/11	11/2	137	310
K.J.	27	0	24/10	21/1	116	348
I.P.	27	0	27/10	29/1	151	343
K.K.	31	0	20/10	23/1	179	292
E.O.	28	1	22/10	28/1	145	280
M.J.	34	1	13/10	25/1	157	268
B.J.	32	0	15/10	30/1	80	330
S.N.	27	5	1/11	17/2	62	353
G.E.	27	1	25/9	22/1	146	385
M.M.	29	1	13/9	10/1	105	374
I.S.	46	2	28/7	20/12	105	315
S.B.	29	1	17/8	9/1	105	306
A.H.	30	1	4/8	28/12	75	349
I.S.	26	1	11/8	8/1	106	443
S.A.	34	0	20/7	20/12	156	362
O.R.	40	2	20/7	20/12	61	323
I.B.	26	0	24/7	28/12	116	426
B.N.	38	0	18/7	23/12	86	459
S.N.	17	0	17/7	13/1	130	405
S.K.	27	0	14/7	8/1	182	416
G.S.	33	0	25/6	23/12	90	457
E.N.	30	2	26/6	28/12	97	429
E.P.	29	2	30/6	2/1	44	484
C.N.	26	0	22/6	27/12	62	444
M.W.	19	0	10/8 ?	13/1	78	480
S.M.	28	1	10/8	13/1	125	457
B.W.	27	0	15/6	28/12	49	504
S.P.	30	0	15/6	2/1	123	446
A.D.	39	0	9/6	28/12	90	491
A.P.	28	0	5/6	28/12	50	445
A.M.	18	0	26/5	28/12	90	502
B.S.	43	1	22/5	2/1	58	499
U.P.	20	0	19/5	2/1	38	568
G.P.	25	0	1/5	27/12	70	496
G.S.	19	0	28/4 ?	27/12	38	475
A.D.	35	2	25/4 ?	21/12	91	488
A.P.	19	0	20/4	20/12	85	522
S.K.	40	7	21/4	23/12	108	624
V.A.	36	4	20/4	23/12	104	517
A.J.	41	2	26/4	23/12	61	624
S.G.	22	0	14/4	21/12	66	582
R.A.	40	1	17/4	27/12	72	466
E.S.	31	0	9/4	21/12	131	512
I.J.	22	2	6/4	21/12	100	567
K.A.	28	1	10/4	27/12	64	556
S.E.	36	1	4/4	28/12	139	440

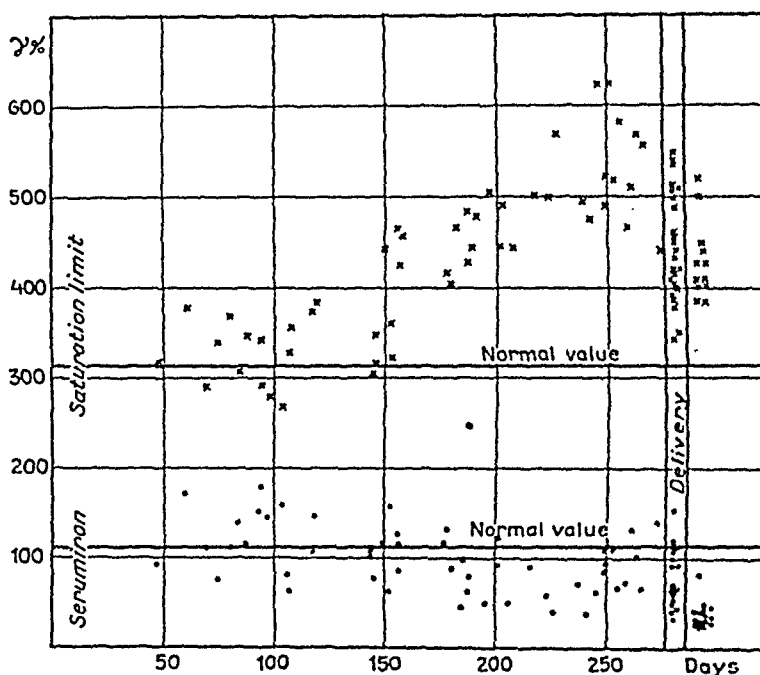


Fig. 7. Variations of the saturation limit and serum iron during pregnancy, delivery and puerperium.

limit go in opposite directions. In the months when the serum iron is normal, the saturation limit is normal too. When serum iron shows a tendency to fall, however, the saturation limit increases, and finally it falls again in the last month of pregnancy, when serum iron shows a tendency to rise. No definite difference can be demonstrated in the value of the saturation limit in the 9' month and at parturition, the material at this stage being too small to afford any reliable evidence in this respect.

During pregnancy the copper content of the serum increases markedly (*e.g.*, Krebs 1928, Heilmeyer *et al.* 1941, Holmberg 1941). In order to ascertain whether there might be any simple connection between the increased saturation limit and the rise in serum copper, Holmberg (not published yet) has determined the serum copper concentration in sera from women in the different months of pregnancy. In this material some sera showed a distinct rise in the copper content without any increase in the saturation limit. Often the serum copper appears to rise earlier in pregnancy than does the saturation limit. These findings rather suggest that there

is no simple connection between the rise in serum copper and the increase in the iron-binding capacity of the serum.

Discussion.

As pointed out, among others, by Vahlquist (1944) the iron requirement of woman during pregnancy does not increase above the normal until the latter half of the gestation. In the first months of pregnancy the fetal and uterine iron requirements is compensated by the absence of any loss of iron with the menses. From about the 5' month of pregnancy the iron requirement increases gradually above the normal level, reaching its maximum towards the end of pregnancy. The iron requirement post partum is subject to wide variation, depending on the loss of blood at the parturition. During the lactation the iron requirement is usually not increased, as the iron given off with the milk does not exceed the normal loss of iron through menstruation. ✓

From the works cited above it is evident that the iron absorption in the early part of pregnancy is about the same as usual, i.e., there is a relative blockade for the absorption of iron. *In the latter part of pregnancy, when the iron requirement of woman is increased, the iron absorption appears indeed to take place more readily than in non-pregnant women.*

On correlation of these physiological facts with the observations reported above on variations of serum iron in the various months of pregnancy, it can be established that as the loss of iron from the organism in general to the fetus and uterus exceeds the normal loss of iron, the serum iron has a tendency to fall to subnormal values. As serum iron decreases, however, the saturation limit is raised, and at the same time the iron absorption proceeds more readily than normally. It seems obvious then to correlate these facts with each other. *In the last months of pregnancy the latent iron-binding capacity of the serum is about twice as great as normally, and serum iron is normal or slightly subnormal, i.e., the quotient $\frac{\text{manifest}}{\text{latent}}$ iron-binding capacity is smaller than normally.* The physiological significance of this may very well be that the lower the quotient the more rapidly is iron eliminated from the intestinal canal and the depots.

Serum Iron and Saturation Limit in Mother and Fetus at Parturition.

Theories about the iron transport from mother to fetus.

It has not been practicable with certainty to establish whether the placenta utilizes the mother's red blood cells or the maternal serum iron as the source of iron for the fetus. Studies reported by Scholten & Veit (1903), Marshall (1932) and Stander (1941) made it reasonable to assume that the red blood cells were subject to phagocytosis in the chorionic villi and to hemolysis by a hemolysin that could be demonstrated in the placenta. After decomposition of the hemoglobin the iron would then be available to the fetus. A weighty argument against this theory was advanced by Pommerenke, Hahn, Bale & Balfour (1942), who gave the mothers radioactive iron by mouth at various points of time prior to the parturition in order to ascertain how soon the iron reached the fetal circulation. In one case, radioactive iron was found in the plasma of the fetus as early as 40 min. after the mother had ingested the iron. In another case, radioactive iron was demonstrated in the red blood cells of the child after 2 hours. In discussing the problem these authors say: »It seems quite improbable that the only route of transmission of iron from the mother to the fetus must be through the complicated chain of events expressed in most texts, i.e., maternal plasma iron to red cell iron, passing of the latter to the placenta, destruction of the red cells with liberation of the contained hemoglobin iron, absorption, and finally elaboration of the iron into hemoglobin of the fetal blood cells». As they found that it takes the radioactive iron only a very short time to pass from the intestinal canal of the mother to the fetal circulation, they assumed »that at least some iron reaching the fetus does not have as its immediate precursor the iron contained in the hemoglobin of the maternal red blood cells». If the transmission of iron from mother to fetus takes place via the maternal serum iron, it is understandable that the radioactive iron can be demonstrated so soon in the fetus.

Serum iron in mother and child at parturition.

Vahlquist, who studied the serum iron level in the mother and fetus in various months of pregnancy and at parturition, found that up to the 5'—6' month of pregnancy the fetal serum iron was lower than the maternal. After this the serum iron in the fetus increases gradually, and in the last two months the serum iron level is higher in the fetus than in the mother. At parturition the fetal (placental) serum iron value is about 160 γ % (Locke, Main & Rosbach 1932, Neuweiler 1938, Thoenes & Aschaffenburg 1934, Vahlquist 1941, Blach & Stoker 1946).

Albers (1941) found the serum iron value in the mothers at parturition to be somewhat higher than observed by other authors; on the other hand, he found the corresponding value in the fetus to be lower than observed by other authors. Thus he stands alone with his view that the maternal and fetal serum iron values lie about at the same level.

The thorough studies of Vahlquist on serum iron in the various months of pregnancy go distinctly against the idea of a simple diffusion balance between the maternal and the fetal serum iron. If the iron is transported from the mother to the fetus via serum iron, the placenta must be able to transmit the iron to the fetus from the maternal organism which has a lower serum iron than the fetus at least during the last months of pregnancy.

Writer's Findings.

As it may be looked upon as an established fact that at the end of pregnancy the fetal serum iron level is higher than the maternal, it seemed of interest to see how the saturation limit behaved in the mother and fetus at parturition. In order to look into this question, determinations of the saturation limit and serum iron level were made on 25 mothers and their newborn children. The results are given in Table 31.

As soon as the child was delivered the umbilical cord was divided, and blood was allowed to drop freely from the placental stump of the cord into a test tube. For technical reasons it was not practicable in every case to withdraw a sample of blood from the mother just at parturition. In those cases where the mothers' blood was not obtained (by venipuncture) within 15 min. after the delivery of the child, the interval (in minutes) between the sampling of the maternal and fetal blood is given in the table.

Table 31.

Comparison between serum in mother (M) and child (F) at parturition. M_o = mean value and M_p = mean value in investigated pairs.

Case No.	Serum protein %		Serum albumin %		Serum bilirubin mg %		Non protein nitrogen mg %		Serum iron γ %		Saturation limit γ %		Blood sampling. M minutes after partur.
	F	M	F	M	F	M	F	M	F	M	F	M	
1.	5.3	7.0	3.8	4.0	2.8	0.3	31	29	132	90	224	442	
2.	5.5	6.4	3.7	4.0	2.1	0.8	31	27	177	113	198	506	
3.	5.8	6.2	3.7	4.0	2.0	0.6	32	29	147	154	260	399	
4.	6.1	6.2	4.1	3.9	2.2	0.3	31	28	130	39	194	346	
5.	6.6	6.6	4.6	4.6	2.0	0.2	32	26	167	62	245	405	
6.	5.8	6.2	4.0	3.6	1.8	0.3	37	26	165	33	210	463	120
7.	4.7	5.8	3.3	3.3	1.6	0.4	27	26	128	47	189	457	90
8.	5.8	6.2	4.0	3.8	2.2	0.4	39	28	177	100	193	419	40
9.	5.9	6.3	4.3	4.3	2.3	0.6	31	28	141	43	255	397	
10.	5.7	6.0	3.9	3.7	2.2	0.6	32	27	207	113	230	344	
11.	5.5	6.4	3.7		1.7		32		145	106	220	514	
12.	5.3	6.5	3.8	4.3	1.8		31		181	92	200	420	
13.	5.7	6.9	4.2		1.7		37		70		224	510	
14.	6.2	7.3	4.0	4.5	1.9		31		142		258	537	
15.	7.8	7.6				0.3	35	26			369	543	
16.	5.7	6.5	3.9	4.7	1.5		35	29	95		297	428	
17.	5.7	6.7	3.9	4.5	1.5		32	30	129	64	234	434	
18.	5.6	6.0	3.9	3.8	1.1	0.9	48	34	137	67	173	454	120
19.	5.5	6.7			1.8	0.5	32	29	169	63	250	509	
20.	5.5	6.5			1.6	0.8	33	29	142	52	266	419	60
21.	6.0	6.9			1.7	0.5	39	31	141	117	201	548	
22.	5.3	7.0	4.0	4.0	1.1						196	440	
23.	5.1	6.2	3.6	4.0	2.2						210	387	
24.	5.9	6.2	4.0		1.6						215	450	
25.	5.0	6.3	3.2		1.7						149	379	
M_o	5.9	6.5	3.9	4.1	1.8	0.5	34	28	147	80	226	446	
M_p	5.7	6.5	3.9	4.1	2.0	0.5	34	28	154	80	226	446	

From Table 31 it is evident that the fetal serum iron concentration on an average is 147 γ % as against the maternal 80 γ %, which is quite in keeping, for instance, with the findings reported by Vahlquist. In the present material it may be that the samples of blood which were withdrawn from the mothers later than 15 min. post partum have given values that really are a little too small, as serum iron normally commences to fall already within the first hours after delivery. Still Vahlquist found even a lower

Table 32.

Serum iron and saturation limit in the puerperium.

Case No.	Age	Loss of blood in parturition	Number of days after delivery	Serum iron γ %	Saturation limit γ %
1.	36	500	3	31	425
2.	23	850	4	81	385
3.	25	350	4	31	384
4.	22	700	4	26	409
5.	19	150	4	40	408
6.	31	100	4	27	425
7.	39	150	4	25	442
8.	19	250	4	38	520
9.	41	450	5	44	339
10.	39	200	5	47	298
11.	33	150	5	36	500
12.	32	1000	5	32	447
13.	29	900	6	33	322
14.	24	125	6	26	408
15.	—	400	6	45	408
16.	20	—	24	30	446
17.	28	—	70	14	420

average value (60 γ %) in the mothers at parturition than was observed in this material, but about the same average value for the children (160 γ %).

On the other hand, in the determination of the saturation limit the mothers were found to give a considerably higher mean value (446 ± 13 γ %) than the children (226 ± 10 γ %).

In the mother thus the iron-binding capacity is nearly twice as high as in the child, while in the child the serum iron concentration is nearly twice as high as in the mother. This goes against the view that the iron-binding protein component of the serum might pass freely through the placenta.

Table 31 gives also the value for non-protein nitrogen in the blood of the mother and child. Generally this value is somewhat higher in the child than in the mother — as was to be expected of a freely diffusable substance that is formed by the fetus but eliminated by the mother.

It is to be pointed out in particular that *the average value for the maternal saturation limit is decidedly higher than the average for normal subjects, while the fetal value is decidedly lower than the normal* — both of these differences being statistically estab-

lished. Noticeable differences are also found in the iron metabolism of the normal subject, the mother and the fetus. Thus the *normal subject* absorbs only an insignificant amount of iron, and makes hardly any use of his iron depots but manages his intermediary iron metabolism by utilizing continually all the iron liberated in the destruction of red blood cells for the synthesis of hemoglobin for new blood cells. The organism of the *mother* has adjusted itself to absorb more iron than normally and to utilize the iron depots in a higher degree than normally in order to make up the loss of iron through the placenta. Finally, the *fetus* employs its supply of iron for synthesis of hemoglobin, it is true, but primarily for filling of the liver depot.

The quotient $\frac{\text{manifest}}{\text{latent}}$ iron-binding capacity is on an average 1.9 in the newborn and only 0.2 in the mother.

Most likely these differences are associated with the circumstance that different phases of the intermediary iron metabolism are encountered in the normal subject, the mother and the fetus — as will be discussed more thoroughly in Chapter VIII.

Chapter VI.

Serum Iron and Saturation Limit after Loss of Blood.

Acute Loss of Blood.

Withdrawal of Blood.

An acute loss of blood is often followed by an incidental rise in serum iron during the first 24 hours, whereafter it falls to a subnormal value. Usually it returns to a normal level only after complete regeneration of blood (for literature, see Brøchner-Mortensen 1943). In view of these observations the findings reported by Hahn, Bale, Ross, Balfour & Whipple (1943) concerning the absorption of iron after an acute loss of blood are of considerable interest. Under normal conditions a relative »mucosal block» for iron is found in dog as well as in man. In the dog an acute bleeding will not increase its capacity for absorption of iron within the first 24 hours; but after this interval the dog commences to utilize the ingested iron in a higher degree than normally, and after one week its iron absorption is 10—20 times greater than before the bleeding.

In order to see whether the saturation limit in man is influenced by an acute loss of 400 ml. blood, a number of donors for blood transfusion were picked out. The experiment was carried out only on persons, who had not given any blood in the last months. Also the persons were excluded who previously had served as donors several times. The serum iron concentration and saturation limit were determined in connection with the venipuncture, whereafter the donors were told to return one week later for a control test. The results are given in Table 33.

Table 33.

Serum iron and saturation limit before and 1 week after withdrawal of blood.

Case No.	Sex	Age	No withdrawal of blood during the last months	Withdrawal of blood			
				Serum iron γ % before	1 week later	Saturation limit γ % before	1 week later
1.	M	27	6	140	116	304	314
2.	M	28	5	107	71	336	347
3.	M	39	5	84	78	307	319
4.	M	23	5	155	84	359	424
5.	F	23	3	121	53	318	352
6.	M	32	9	88	82	304	420
7.	F	21	4	84	37	405	450
8.	M	34	6	124	112	314	364
9.	M	26	11	102	55	312	332
10.	M	34	2,5	122	152	330	326
11.	F	20	—	128	103	313	365
12.	M	33	5	145	84	334	364
13.	M	26	—	192	104	258	351
14.	F	27	5	162	69	305	305
15.	M	30	5	60	48	291	287
16.	M	30	5	112	59	323	442
17.	F	47	6	68	36	345	391
Mean value:				117	79	321	362

From Table 33 it will be noticed that prior to the loss of blood the average serum iron was 117 γ %, and after one week it had fallen to 79 γ %. This post-hemorrhagic fall in serum iron was more or less pronounced in 16 of the 17 subjects examined. The saturation limit, however, was found to change in opposite direction — with an average value of 321 γ % before the loss of blood, and 363 γ % after one week. In 4 cases the values thus obtained exceeded the highest values found in the normal material. The mean value obtained one week after the loss of blood ($M=363 \pm 12$ γ %) is also significantly higher than the mean value for the normal material (315 ± 3.3 γ %). This rise in the saturation limit is not characteristic of the entire material, however, as 6 of the 17 subjects examined showed values, the deviation of which from the initial value falls within the limits of experimental error.

Thus the results indicate that *while the serum iron increases after an acute loss of blood* — in connection with the regeneration of blood — *the saturation limit has a tendency to rise.*

The quotient $\frac{\text{manifest}}{\text{latent}}$ iron-binding capacity before the bleeding was about 0.6, and one week later it was 0.3.

Saturation Limit after Gastrointestinal Hemorrhage.

After a large loss of blood the saturation limit would reasonably be expected to fall because of the loss of plasma if the organism replaces the iron-binding component at the same rate as other plasma proteins. In order to get an idea about the variations in the saturation limit associated with, and subsequent to, an acute loss of blood, a number of patients with hemorrhages from peptic ulcer were examined. The results are given in Table 34.

The material is not uniform as the patients were admitted to the hospital at varying junctures after the hemorrhage. The first 7 cases indicate that the saturation limit decreases in connection with large acute hemorrhages. Two patients (Nos. 8 and 9) showed on admission a slightly hypochronic color index, which indicates that they were not hospitalized at the first attack of hemorrhage. In contrast to the other patients, these two showed also a high value for the saturation limit already on the admission to the hospital.

In all the patients there was a tendency to a rise in the values for the saturation limit after the hemorrhage had ceased. During the period in which the patients were observed, however, this rise in the saturation limit was neither rapid nor particularly pronounced.

Chronic Loss of Blood.

After Repeated Service as Donors.

On 200 donors Fowler & Barer (1942) have investigated the rapidity with which hemoglobin is reproduced after a loss of about 550 ml. blood. The average time required for attainment of the initial hemoglobin value was 49.6 days. The rate of regen-

Table 34.

Saturation limit in patients with gastro-intestinal hemorrhage.

Case No.	Sex	Age	Hb %	R. b. c. mill./cmm.	Serum iron γ %	Saturation limit γ %	Date
1.	M	20	49	2.8	22	276	4/12
			58	3.2	19	313	12/12
			65	4.0	28	329	17/12
2.	M	70	55	3.3	53	230	20/1
			66	3.5	57	274	27/1
3.	F	46	56	2.6	86	280	10/12
			70	3.6	108	328	17/12
4.	M	66	42	2.4	20	300	3/11
			52	3.4	26	332	23/11
5.	F	45	51	3.0	26	260	15/1
			53	3.1	29	266	21/1
			63	3.2	23	264	27/1
6.	M	60	40	2.3	42	233	20/11
7.	M	80	55	3.7	25	314	22/1
			48	2.8	14	280	
8.	M	44	34	2.2	23	358	27/11
			37	2.4	10	395	4/12
			40	2.5	31	480	12/12
			47	3.5	31	395	16/12
9.	F	51	53	3.4	11	438	15/1
			55	3.4	44	416	21/1
			66	3.7	62	400	27/1

1. Duodenal ulcer with hemorrhage. Acute onset with hematemesis on 22/11.
2. Duodenal ulcer with hemorrhage. 12/1: Hematemesis.
3. Duodenal ulcer with hemorrhage. 28/11: Hematemesis.
4. Duodenal ulcer. Probably silent hemorrhage at home.
5. Duodenal ulcer with hemorrhage. 9/1: Hematemesis.
6. Duodenal ulcer with hemorrhage. Terminating fatally.
7. Gastric ulcer with hemorrhage. Subacute onset about 15/1.
8. Acute tiredness on 10/11. Admission 2 weeks later. Blood test on feces positive.
9. Duodenal ulcer with hemorrhage. Onset on 12/1.

eration was somewhat more rapid for men (0.049 g. % hemoglobin/24 hours) than for women (0.040 g. % hemoglobin/24 hours).

In order to ascertain the influence of frequent loss of blood on the saturation limit, a number of persons were examined who

Table 35.

Saturation limit in persons who repeatedly have served as blood donors.

Case No.	Sex	Age	Total loss of blood (liters) from withdrawal of blood	Loss of blood during last year (liters)	Period since last withdrawal of blood (months)	Hb %	R.b.c. mill/cmm	Serum iron γ %	Saturation limit γ %
1.	F	30	3	2	3	84	4.8	76	353
2.	F	27	6	2	2			48	370
3.	M	23	2 1/2	2	2.5	93	4.9	(242)	340
4.	M	42	19	3	2	86	4.4	87	361
5.	M	22	3	2 1/2	2	90	4.7	63	296
6.	M	30	3	3	2			49	363
7.	M	23	3	3	2	87	4.7	141	301
8.	M	29	3	2 1/2	2	86	4.9	66	371
9.	M	33	2 1/2	2 1/2	2	89	4.4	130	401
10.	M	36	4	1 1/2	2	84	4.4	55	347
11.	M	37	2 1/2	2 1/2	2	92	5.1	40	417
12.	M	38	3	3	2.5	105	5.3	86	356
13.	M	21	6	3	1.7	90	4.9	56	421
14.	M	27	2	2	2			70	329
15.	M	27	2 1/2	2 1/2	2	80	4.0	75	295
16.	M	47	2 1/2	2 1/2	1.5			(251)	357
17.	M	36	3	3	1.5			77	483
Mean value:								(95) 75	362

had served as donors repeatedly in the past year. On repeated loss of blood the organism will have to regulate its iron metabolism so that the iron is absorbed more readily than normally — if the organism is not to get poor in iron. For, according to Hahn (1937), the storage iron is only sufficient to replace about 30—50 % of the normally circulating hemoglobin. In several of the subjects here examined the losses of iron through the repeated venipunctures (about 400 ml. blood each time) have been so large that the storage iron fraction has not been sufficient to replace the loss of iron. The present withdrawal of blood was made at a time after the last venipuncture when the hemoglobin level should have become normal again if the rate of regeneration had been normal. The results are given in Table 35.

The hemoglobin values and red blood counts found in a majority of these cases are slightly subnormal. This applies also

to serum iron, which in 13 of the 17 examined donors was under 100 γ $\%$. Of the remaining subjects, 2 showed perfectly normal values, while 2 gave abnormally high values.¹ Largely, the values here obtained for serum iron agree with those observed by Sköld & Waldenström (1940) in persons who had given blood several times within one year. In their material, however, there is no case with abnormally high values. The mean value for the saturation limit in our material is 362 ± 12 γ $\%$ as against the normal value of 315 ± 3.3 γ $\%$. The difference is statistically significant.

That the saturation limit may rise after repeated venipunctures is also indirectly evident from the observation reported by Vahlquist (1940). He performed a peroral iron tolerance test on a male subject who in the last 12 weeks had furnished blood 3 times, the last of which was 3 weeks before. This subject now showed a rise in serum iron of over 400 γ $\%$, which naturally implies that his saturation limit must have lain at an abnormally high level.

The writer's material shows no decided connection between variations in hemoglobin, serum iron and saturation limit. It may be pointed out merely that serum iron shows a tendency to lower values than normally, while the saturation limit has a tendency to values above the normal.

Sideropenic Anemia.

In recent years the term »sideropenic anemia» has become a collective designation for anemias that may be abolished by means of an adequate iron therapy. The cause of the iron deficiency may vary: increased loss of iron (pregnancy, chronic hemorrhage), difficulty in iron absorption (achylia), or decrease in the iron supply (diet poor in iron). This group of anemias should also include the so-called »essential sideropenic anemia» the pathophysiological basis of which is still obscure. A constitutional factor appears also to be of importance to the appearance of sideropenic anemia (Lundholm 1939).

In the writer's material, chronic hemorrhage has been the predominant cause of sideropenic anemia. The variations in the

¹ It cannot be excluded that these persons may have taken iron.

Table 36.

Saturation limit in patients with chronic hemorrhagic anemia.

Case No.	Sex	Age	Hb %	R. b. c. mill./cmm	Sedimentation rate mm/hour	Serum iron γ %	Saturation limit γ %	Date
1.	F	46	59	3.0	11	38	455	
2.	F	38	58	2.9	15	25	400	
3.	F	40	55	4.4	15	24	430	
4.	F	61	52	3.6	8	47	406	
5.	F	46	31	2.6	5	16	388	1/4
			50	3.8		20	435	14/4
6.	M	41	58	4.3	8	58	445	25/1
			69	4.7		52	490	4/2
7.	F		56	3.0	10	32	383	
8.	M	33	57	3.7	20	29	564	23/11
						53	392	12/12
			85	4.9		47	380	18/12
			58	3.6		28	458	9/1
			70	4.0	12	47	483	15/1
			73	4.1		70	482	22/1
			76	4.2		52	420	19/2
9.	M	50	34	3.0	19	26	436	
10.	F	66	36	2.9	48	38	440	
11.	F	35	60	3.1	12	32	378	

1. Cystic hemorrhagic metropathy.
2. Myomata of uterus with menorrhagia.
3. Cervical polyp of uterus with menorrhagia.
4. Metrorrhagia; duodenal ulcer.
5. Increasing tiredness during the last year. Menstruation more frequent than previously. Pronounced sideropenic epithelial symptoms. Iron therapy.
6. 2 weeks before admission, shortness of breath and anxiety on working. No source of bleeding established by the examination. Iron therapy.
7. Cesarean section 1 year before. Since then, abdominal trouble.
8. Changes in the blood values (Hb. and r.b.c.) show that the patient has had 2 intestinal hemorrhages, one before admission, the other at home between ¹⁸/₁₂ and ⁹/₁. During both periods, Weber positive. Autopsy diagnosis (problaparatomy): Schaumann's disease; cirrhosis of liver (Laennec).
9. Weber positive repeatedly. X-ray diagnosis: Cancer of colon.
10. Last 6 months, increasing tiredness. Palpable tumor of stomach. Weber strongly positive.
11. Menometrorrhagia. For several years, profuse bleedings.

Table 37.

Case No.	Sex	Age	Hb %	R. b. c. mill./cmm	Sedimentation rate mm/hour	Serum iron γ %	Saturation limit γ %
1.	F	38	65	3.2	7	18	390
2.	F	81	57	3.2		36	374
3.	F	18	47	3.6	28	13	290
4.	F	64	68	3.7	1	86	196

1. Complaints of sterility and amenorrhea. Past history negative as to cause of sidropenia.

2. Last 6 months, more pale; loss of weight. Complaint of hoarseness. Recurrens paralysis.

3. Tiredness; muscular rheumatism; sidropenic epithelial symptoms. Menses normal. Free hydrochloric acid in the stomach juice.

4. 6 months ago, serum iron 164 γ % (Hb 90 %; r.b.c. 4.5/cmm). All typical sidropenic epithelial symptoms, including marked hypopharyngeal stenosis. Treated with iron and vitamin B. Stenosis persisting, and anemia has developed.

saturation limit in connection with the increased iron requirement during pregnancy has been dealt with already in Chapter V. In Table 36 the results are given of serum iron and saturation limit determinations on patients with anemia after chronic hemorrhage. Whether the hemorrhages have brought about the anemia in all the cases could not be decided with certainty, of course, but at any rate they must have constituted a contributory factor.

As was to be expected, *the serum iron value was low in all the cases. In contrast hereto, the saturation limit was high.* Patient No. 8, who was under observation for a considerable length of time, shows how the values for the saturation limit increase after the hemorrhages and then fall off again towards the normal level when the hemoglobin values begin to turn normal.

In all these cases the quotient $\frac{\text{manifest}}{\text{latent}}$ iron-binding capacity was less than 0.2.

So, in latent or manifest iron deficiency resulting from considerable loss of iron (pregnancy or repeated loss of blood) the values for the saturation limit rise above the normal level. These conditions are also characterized by the fact that iron given by mouth is absorbed more readily than normal.

Table 37 gives a schematic survey of some patients with sideropenic anemia due to causes other than chronic hemorrhage. Cases 1, 2 and 3 may possibly belong to the group of essential sideropenic anemia even though patients Nos. 1 and 2 appear to have normochromic anemia (analytical error?). Cases 1 and 2 show the same relation between the serum iron concentration and the saturation limit as was seen in cases of chronic hemorrhagic anemia. Case 3, which is the only unquestionable case of chlorosis here observed, shows a normal value for the saturation limit. Case 4 (see clinical remarks) gives a low value for the saturation limit. More likely, however, this patient was not suffering from sideropenia but possibly from riboflavin deficiency.

Chapter VII.

Serum Iron and Saturation Limit in other Diseases.

Diseases with Increased Intravital Hemolysis.

Pernicious Anemia.

In untreated pernicious anemia the serum iron concentration is usually high (175—300 γ %). On institution of liver therapy it rapidly decreases to subnormal values. Thus a pronounced fall in serum iron is usually observed as early as 1—2 days after intramuscular injection of a liver preparation.

Waldenström (1944) has shown clearly that the rise in serum iron after intravenous injection of iron (10 mg.) is lesser in patients with untreated pernicious anemia than in normal subjects. After the liver therapy had been commenced and the serum iron values had fallen, he found a normal rise in serum iron when the intravenous injection of iron was repeated.

Writer's Findings.

The serum iron concentration and saturation limit were determined on patients with pernicious anemia before and during liver therapy. The results are given in Table 38.

The values for serum iron are quite in keeping with those obtained by other investigators before and after institution of liver therapy.

The mean value for the saturation limit at the commencement of the treatment was 236 ± 9 γ %. The difference between this and the mean value for the normal material (315 ± 3.3 γ %) is significant.

Table 38. *Saturation limit in pernicious anemia.*

Case No.	Sex	Age	Days after beginning of liver therapy	Hb. %	R. b. c. mill./cmm.	Serum iron γ %	Saturation limit γ %
1.	M	61	0	40	1.5	167	157
			5			48	152
			12	62	2.6	31	170
2.	M	66	0	46	1.9	219	200
			2			196	187
			3			139	180
			12	53	2.5	58	217
3.	M	80	0	28	1.0	128	200
			7			20	152
			12	40	1.7	18	165
			24	56	2.7	18	200
4.	F	76	3	53	2.0	29	210
5.	M		0	39	1.5	193	210
6.	M	56	0	51	1.7	204	236
7.	M	69	1	36	1.3	169	242
			3			63	250
8.	M	48	0	33	1.9	197	245
			4			136	256
9.	M	75	0	40	1.5	206	245
			4			42	250
10.	M	72	0	32	1.2	253	248
			2			58	246
			7			38	230
			18			33	210
			27	48	3.4	36	255
11.	F	58	0	79	3.5	113	250
			3			136	240
12.	F	62	0	52	1.9	203	256
			2 1/2			22	230
13.	F	58	0	60	2.0	202	260
			4			98	244
			16			22	222
			22	73	2.6	28	248
14.	F	56	I II				
			0	27	1.1	187	262
			1			120	248
			4			46	256
			8			188	290
15.	M	62	15 3	40	2.0	20	280
			0	12	0.6	285	265
			7	27	1.5	16	164
			18			25	229
16.	M	53	26	51	2.9	20	243
			0	21	0.8	281	285
			3	27	1.4	28	180
			30	58	2.7	20	280
17.	M	45	0	78	3.4	72	182
			4			22	196
			20	86	3.7	70	255

Five patients gave serum iron values that practically were equal to the values for the saturation limit. In some cases the serum iron concentration was even higher than the corresponding saturation limit. These differences are so small, however, that they are not established — in view of the analytical error for the determinations.

Clinically the serum iron values are taken as an expression of the actual phase of the iron metabolism. When a patient shows a serum iron concentration of 150 γ %, this is usually taken as a perfectly normal value. But this is correct only if the saturation limit is about 300 γ %. If we find a serum iron value of 150 γ % in a patient suspect of pernicious anemia, a determination of the saturation limit may possibly reveal that the serum iron value is not at all to be looked upon as normal but actually as a maximal value for the patient in question. As a matter of fact *a low value for the latent iron-binding capacity is even more characteristic of the untreated pernicious anemia than is a high serum iron concentration.*

After the institution of liver therapy the serum iron concentration falls to low values without the saturation limit being influenced in any particular degree (exceptions to this rule are seen in Cases 3, 15 and 16 in Table 38). This has been pointed

Table 38.

1. Reticulocyt. max. (129 ‰) on 5' day.
2. Relatively inactive test preparation. Reticulocyt. max. 15 ‰.
3. 2'—5' day, fever about 38—39° as reaction to liver therapy. Reticulocyt. max. (188 ‰) on 6' day.
4. Reticulocyt. max. (89 ‰) on 3' day.
7. Reticulocyt. max. (169 ‰) on 6' day.
8. Reticulocyt. max. (89 ‰) on 6' day.
9. Reticulocyt. max. (180 ‰) on 6'—7' day. Complication: Polyp. malign. of stomach.
10. Reticulocyt. max. 368 ‰ on 6' day.
11. The patient had been treated earlier with success.
12. Reticulocyt. max. (187 ‰) on 5' day.
13. Reticulocyt. max. (92 ‰) on 6' day.
14. The patient was treated with a test preparation. First reticulocyt. max. (152 ‰) on 6' day. Second reticulocyt. max. (96 ‰) on 5' day.
15. On 2'—6' days fever about 40° in response to liver therapy or blood transfusions. Reticulocyt. max. (104 ‰) on 10' day.
16. On 2'—5' days fever, about 39—40° in response to liver therapy or blood transfusions. Reticulocyt. max. (147 ‰) on 8' day.
17. Achrestic, anemia. No response to liver therapy, but to folic acid. Reticulocyt. max. (69 ‰) on 6' day.

out previously by Holmberg & Laurell (1945). Some of the patients show a slight fall in the saturation limit, but this appears later than the decrease in serum iron, and it is followed again by a distinct rise towards the normal level.¹ Cases 3, 15 and 16 are of interest because the values for the saturation limit here decreased markedly in the beginning of the treatment. These patients were in a poor condition. In the beginning of the treatment they responded with fever (about 40°) for a couple of days (in Cases 3 and 15, besides injection of the liver preparation, blood transfusions were given too). As will be shown in the following, usually the saturation limit is lowered markedly in febrile infections. Most likely, the fall in the saturation limit in Cases 3, 15 and 16 is attributable to the fever reaction.

Other Diseases with Increased Intravital Hemolysis.

Table 39 gives a survey of the saturation limit in 6 patients suffering from hemolytic anemia other than pernicious anemia. As pointed out already, usually the saturation limit is lower than normal in patients with pernicious anemia. Also in these 6 patients, with hemolytic anemia the values for the saturation limit are lower than normal. Still, this material is all too small and non-homogeneous to allow of any conclusion in this respect.

On increased hemolysis there is also an increase in the amount of globin liberated through the decomposition of hemoglobin. As the saturation limit does not rise with increasing hemolysis, the iron-binding component in serum is not very likely to be identical with globin.

Diseases of the Liver.

Acute Hepatitis.

In the acute hepatitis the serum iron concentration is usually found to be high during the first weeks of illness (for literature,

¹ The patients left the hospital before complete hematological restitution was attained, and hence, unfortunately, the present material furnishes no data on the value for the saturation limit in treated patients with a normal blood picture.

Table 39.
Saturation limit in hemolytic anemia.

Case No.	Sex	Age	Date	Hb %	R. b. c. mill./cmm	Serum bilirubin mg %	Serum iron γ %	Saturation Limit γ %
1.	M	67	24/1	36	1.7	2.8	159	188
			3/2	34	1.5	1.6	201	205
			10/2	30	1.3		190	202
					seven blood transfusions			
			38/2	53	2.6		254	280
2.	F	69	21/11	27	1.4	0.5	252	276
					blood transfusions			
			26/11	34	1.7	0.5	113	259
3.	M	50	13/11	66	3.7	2.7	92	234
			23/11	77	3.9		114	230
4.	F	70	5/10	34	1.5	6.2	74	240
					blood transfusions			
			9/10	38	1.75	7.2	110	115
5.	F	60		42	2.5	2.9	49	245
6.	M	58	4/12	97	4.8		11	220
			10/12	82	4.3		76	240
			18/12	76	3.9		98	246

1. Increasing tiredness for half a year. Temperature 37—38°. Reacts with max. 20 % reticulocytes on liver therapy. Bone marrow: Rich in cells. No pathological cells.

2. Exitus. Autopsy diagnosis: Toxic anemia of hemolytic type.

3. Acute onset on 8/11. Typical hemolytic icterus with increased osmotic fragility.

4. Increasing tiredness for the last 6 months. In the hospital 30—50 % reticulocytes. Sedimentation rate 154 mm./hour. Temperature about 38°.

5. Splenomegaly. Tiredness and icterus for the last 6 months. 100 % reticulocytes without any therapy.

6. Dementia paralytica. Malaria therapy. Temperature: 4/12 — 40°, 10/12 — 40.2°, 18/12 — 37°.

see Vahlquist [1941] and Brøchner-Mortensen [1943]). The highest values for serum iron are found about simultaneously with the highest values for bilirubin or after the latter have commenced to decrease. Often the serum iron keeps at a high level also for some length of time after the bilirubin value has commenced to fall.

Several theories have been advanced concerning the cause of the rise in serum iron in acute hepatitis. As a large part of the storage-iron fraction of the organism is located in the liver in

the form of ferritin, it seems rather reasonable to assume that the rise in serum iron during the acute phase of the hepatitis is due to liberation of iron from the liver cells, and that the regulatory influence of the liver upon the serum iron level is put out of function.

The results that have been published from peroral, intramuscular and intravenous iron tolerance tests on patients with acute hepatitis suggest that occasionally changes may appear also in the iron-binding component content of the serum. Waldenström (1944) has presented his own results together with some unpublished investigations by Uhnöo which show that after peroral administration of iron to patients with acute hepatitis showing a serum iron concentration over 200 γ ‰, the rise in serum iron is just as high as in normal subjects — in a few patients it even rose above 400 γ ‰ (maximal value observed, about 600 γ ‰). In two patients Bröchner-Mortensen (1942) found a serum iron value of about 450 γ ‰ after intravenous injection of 10 mg. iron.

Presumably these high serum iron values have been due to an abnormally high saturation limit in the respective patients. In one case of acute hepatitis Holmberg & Laurell (1945) found the saturation limit of 690 γ ‰.

Writer's Findings.

In order to see whether the saturation limit might show any regular changes in the course of the lesion, the saturation limit, serum iron and bilirubin were determined repeatedly on 10 patients suffering from acute hepatitis. The findings are recorded in Table 40.

From the laboratory notes on the course of the disease in the individual cases (Table 40) it is evident that in most of the patients the highest values for serum iron were found about simultaneously with the maximal values for bilirubin. In 3 of the 10 cases the highest serum iron value was not obtained until the bilirubin values had commenced to fall. No definite connection can be established between the height of serum iron and the bilirubin concentration. Thus the variations in serum iron

Table 40. *Saturation limit in acute hepatitis.*

Case No.	Day of illness	Serum		Saturation limit γ %	Thymol (Ex)	Serum		Takata	
		bili-rubin mg %	iron γ %			citric acid γ/ml.	phosphatase units		
1.	5	8.2	156	302	0.51	37	18	neg.	
	10	2.1	193	350	0.74				
	15	1.9	198	375					
	29	0.2	46	390	0.07				
2.	7	3.3	204	517	0.32	43	20	neg.	
	11	2.5	167	515					
	36	0.9	105	532	0.17				
3.	5	8.4	135	285	0.39	39	13	neg.	
	10	8.6	177	290					
	16		180	320					
	49	0.7	111	274					
4.	14	6.5	261	438	0.26				
	34	0.9	124	366	0.06				
	40	2.8	172	374	0.05				
	48	2.8	166	405	0.50				
5.	7	7.4	165	307	0.89	24	6		
	12	8.2	167	307		29	7.5		
	22	3.6	131	349					
6.	10	1.8	178	366		35	10		
	16	1.0	109	394					
7.	7	2.1	124	307	0.19	28	12	neg.	
	13	0.9	210	392	0.20				
	20	0.9	166	400	0.30	25			
	27	0.5	135	385	0.15				
	37	0.5	65	380	0.14				
	46	1.6	108	370					
	60	0.7	184	344	0.13				
8.	4	8.6	243	290	0.23	30	9	neg.	
	27	6.5	252	301	0.23				
	32	4.4	201	290	0.08				
	39	2.9	163	376					
	48	2.1	180	350	0.07	26	7.5		
	54	1.5	208	370					
	69	1.0	125	336					
9.	5	7.4	241	233	0.54	42	7	pos.	
	8	7.6	145	223					
	12	8.7	139	241					
	17	4.2	175	280					
	22	2.3	166	276		26	8		
	29	1.5	125	292	0.60				
	69	0.8	157	335	0.57				
	99	0.8	147	365	0.47				
	115	0.6	101	356					
10.	11	10.6	217	233	0.53	40	10		
	17	8.2	244	357					
	22	6.4	277	393					
	34	1.8	179	362					
	46	1.3	110	340					
	53	1.2	103	295					

and serum bilirubin in the patients here examined are quite in keeping with the findings reported by previous investigators.

The material is too small, however, to allow of any definite conclusions as to variations in the saturation limit in the course of acute hepatitis. Still, it is plainly evident that the values for the saturation limit change in the healing phase of the disease.

All the patients were examined in 4—14 days after the first symptoms of the disease had been noticed. In two cases, Nos. 9 and 10, the value for the saturation limit was subnormal at the first examination. Two patients, Nos. 2 and 4, showed abnormally high values, while the remaining 6 values at the first examination fell within the normal limits of variation.

In all the cases — perhaps with the exception of No. 4 — there was a more or less protracted rise in the saturation limit in connection with the improvement of the patients. Usually the maximum values for the saturation limit did not appear until the values for bilirubin and serum iron had commenced to fall and the disease was regressing. It is to be noted in particular that Case 9, which was the most protracted in this material, showed at first a low value for the saturation limit which gradually returned to a normal level but slowly.

At some time in the course of the disease, *most of these cases showed a saturation limit that was higher than is usually seen in normal subjects*; and this explains why previous authors sometimes have found unusually high values for serum iron in intravenous or peroral iron tolerance tests on this kind of patients.

Cirrhosis of the Liver.

Table 41 gives the findings in 5 patients suffering from cirrhosis of the liver and 1 patient suffering from recurrent hepatitis with poor tendency to healing. The last-mentioned patient (1) showed a distinct decrease in the saturation limit during the progressive stage of the disease, with a slow return to normal level on occasional improvement. The Takata-Ara test showed pathological flocculation throughout.

In all the five patients with cirrhosis of the liver the saturation limit was distinctly lowered.

Table 41.
Saturation limit in cirrhosis of the liver.

Case No.	Day of illness	Serum		Saturation limit γ %	Thymol (Ex)	Serum		Takata	Hb o/o	R.b.c. mill./ cmm
		bili- rubin mg %	iron γ %			citric acid γ/ml.	phosphatase (E)			
1.	15	3.4	83	250	0.44	54	40	++	73	3.5
	22	4.1	76	232						
	36	2.1	86	294						
	56	1.8	78	280						
	86		155	255	0.05				79	4.0
	145	2.8	184	250						
	210	5.3	157	210	0.52	34	75	++	84	4.2
2.		2.2	85	181	0.59	43	11	++	81	3.9
3.		0.6	39	105	0.11			++	43	2.1
4.			45	152					58	2.8
5.		0.7	50	148	0.26	27	6	++	78	4.0
6.		4.4	134	152	0.28	37	9	++	82	3.8
		2.1	92	175	0.37		7			

1. Acute hepatitis 1 year ago.
2. Acute hepatitis about 1 year ago.
3. Hepatosplenomegaly. Abdominal complaints for 3 years.
4. Diagnosis at autopsy: Cirrhosis of liver.
5. Half a year ago, icterus and fever. Now ascites.
6. Chronic alcoholism for at least 10 years. Increasing tiredness during the last year.

Acute and Chronic Infections.

It has long been realized that patients with severe acute or chronic infections often develop anemias that are refractory to iron therapy. A great many investigations have been carried out to ascertain the cause of these infection anemias.

Robscheit-Robbins & Whipple (1936) showed that in an anemic dog the blood regeneration was inhibited by intramuscular injection of turpentine resulting in an aseptic inflammatory process.

In particular, Heilmeyer (1937—1941) has investigated the iron metabolism in cases of infection and intoxication. Acute infections and intoxications are associated with a very rapid fall in serum iron, which does not return to a normal level until the inflammatory condition has subsided. Heilmeyer (1937) advanced

the hypothesis that the iron requirement of the reticulo-endothelial system was increased during infections. He took the appearance of the anemia to be a secondary phenomenon resulting from the low serum iron level in inflammatory conditions. Schäfer (1942) was able to show, however, that the serum iron level could almost be normalized through intensive iron therapy also in the infectious state of the patient though without the anemia being abolished. Schäfer (1942) also studied the iron metabolism in infected mice, and found that such animals absorbed more iron than normally; but this iron was not used for synthesis of hemoglobin. Instead, he found an increase in the iron content of the liver and the spleen. Also Sandberg & Holly (1942) found the iron content of the liver and spleen to be increased in connection with infections.

In connection with their own investigations, Hemmeler (1946) and Cartwright, Lauritsen, Jones, Merrill & Wintrobe (1946) have reviewed the literature on infection anemias. Hemmeler points out that the infection anemias are normochromic or slightly hypochromic, that they appear more rapidly in diseases with high fever, and that they are not influenced by iron therapy. »As soon as the infectious disease is cured, regeneration of the blood is very pronounced, a phenomenon which is characterized in the blood by marked reticulocytosis.» »Comparative studies of the blood and marrow permit the conclusion to be drawn that the infectious anemia is consecutive to an insufficient neoformation of red corpuscles, provoked by a disturbed maturation of the erythropoiesis.»

Hahn, Bale & Whipple (1946) found that when radioactive iron is given by mouth to an animal in which a terpine abscess is developing, the amount of radioactive iron found in the red blood cells in the following days is less than would be expected. On this account they assumed that in inflammatory conditions the absorption of iron is lower than normally. Dubach, Moore & Minnich (1946) have pointed out, however, that the amount of radioactive iron found in the red blood cells after ingestion of this form of iron cannot be taken as an expression for the amount of iron absorbed in the inflammatory condition because the erythropoiesis in the bone marrow then is impaired.

In experiments with radioactive iron Cartwright and collaborators (1946) have shown that in infectious conditions iron is eliminated from the blood plasma more rapidly than normally. »As a result of inflammatory reaction, iron is diverted to the tissues and is not made available for hemoglobin synthesis.» Greenberg, Wintrobe *et al.* (1947) have shown in experiments with radioactive iron on pigs that »the anemia of infection is caused by impaired hemoglobin production». These authors (1947) also gave radioactive iron intravenously, drop by drop, to patients with chronic infections but were unable in this way to find any increase in the erythropoiesis. »Since even very intensive iron therapy is ineffective», they say, »it follows that iron is not the limiting factor in the production of the anemia, even though diversion of iron from the plasma does occur». These authors also injected radioactive iron into infected rats on which autopsy was performed 3—4 days later. The injected iron was then found chiefly in the liver and spleen, but only an insignificant amount in the inflammatory foci.

Holmberg & Laurell (1945) determined the saturation limit on some patients with various infectious processes, and found the values in these cases to be subnormal.

Greenberg, Wintrobe *et al.* (1947) have also investigated the variations in serum iron when iron was injected intravenously into patients with some infectious lesions. They found »that in both the normal subject and in the patient with infection, some mechanism acts to prevent the iron level from rising to the expected values after injection of iron. In the patient with infection this 'brake' apparently acts at a lower level of iron.» Thus, also this observation indicates indirectly that the saturation limit in infections is lower than normally.

In order to elucidate the intermediary iron metabolism in acute and chronic infections the serum iron and saturation limit have been determined in various infectious conditions.

Acute Infections.

The findings obtained are reported in Table 42, from which it is evident that the serum iron was low in all the patients

Table 42.
Saturation limit in acute infections.

Case No.	Sex	Age	Sedimentation rate mm/hour	Day of illness	Temperature °	Serum iron γ %	Saturation limit γ %
1.	M	35	32	4	38	12	186
				7	37.2	56	253
				11	36.8	65	296
2.	M	38	80	15	38	28	165
				28	37—38	77	259
				48	37	92	256
3.	M	43	49	9	38.5	18	175
				13	37.8	45	165
				17	37.4	68	204
4.	F	24	63	4	38.6	10	216
				9	37	8	220
				13	37	18	262
5.	F	74	14	30	38.5	20	180
				45	38—39	36	149
				60	37—38	62	178
6.	F	70	31		37.2	30	186
7.	M	61	6	(5)	37—40	21	132
8.	M	52			38.4	20	208
9.	M	43	101	12	39	35	155
10.	F	22	22	(2)	39	12	196
11.	F	59	65		37.2	71	88
12.	F	68	28	30	37	63	230
13.	F	54	35	2	39.2	42	245
14.	F	18	45	6	38.8	18	210
15.	M	27	57	14	40.8	39	140

1. Lobar pneumonia, right. Temperature 40° on 2' day of illness, 39° on 3' day.

2. Lobar pneumonia, left, with myocarditis. Temperature about 38° for 3 weeks.

3. Atelectasis, left lung. Slow return of temperature to normal level.

4. Lobar pneumonia.

5. Fever from unknown cause.

6. Softening of the brain; Femoral thrombosis; Pulmonary embolism(?) (11 days earlier, temperature 39°.)

7. For 5 days: Temperature varying between 37° and 40°. Serum protein 4.4 %.

8. Postoperative febrilia (after pulmetomy).

9. Lobar pneumonia, right, with empyema. Temperature 40—39° for 11 days.

10. Uterine abrasion for metrorrhagia, 2 days earlier.

11. Pleuropneumonia, right; Chronic nephritis; chronic polyarthritis. Under convalescence after pneumonia, the patient had a relapse and has just become afebrile through penicillin therapy.

12. Thrombosis of left femoral vein.

13. Rheumatic fever with acute tonsillitis.

14. Exudative pleurisy (tuberculous).

15. Sepsis (pyocyaneus) after operation.

examined. Further, it ought to be noticed that in the present material *the values for the saturation limit were generally lower than normally*. No absolute connection was found between the temperature of the patient and the value of the saturation limit, even though the lowest values generally were found in patients with a relatively high temperature.

On comparison between the values obtained for serum iron and the saturation limit in this material and the normal material it is found that *the serum iron and the saturation limit appear to be lowered to about the same extent in infections*. This implies that the value for the latent iron-binding capacity of the serum does not change in any particular degree. In acute infections thus the type of fall in serum iron differs from that observed in increased erythropoiesis, as regeneration of the blood is not associated with any fall in the saturation limit (increase in the latent iron-binding capacity).

It has been ascertained that the serum iron concentration falls markedly already in the first days of fever (*e.g.*, Heilmeyer 1937). It has not been possible to decide conclusively whether the fall in saturation limit chronologically accompanies the decrease in serum iron. Still, the saturation limit appears to fall chiefly during the high-febrile stage of the infection. Later, when the infection subsides and the temperature falls, the value for the saturation limit returns again to a normal level though more slowly than the temperature (Cases 1—5).

Why the value for the saturation limit falls in connection with an acute infection is still unsettled. This decrease may either be due to inhibition through the infection of the formation of iron-binding component, or the entire serum iron-protein complex may leave the blood stream in acute infections in order to meet some functional requirement in the combating of the infection by the organism.

Chronic Infections.

In order to see whether the saturation limit falls also in patients suffering from chronic infections, a number of patients with chronic polyarthritis were picked out as well as patients with pulmonary tuberculosis.

Table 43.
Saturation limit in chronic rheumatic polyarthritis.

Case No.	Sex	Age	Hb %	R.b.c. mill./cmm	Sedimentation rate mm/hour	Serum iron γ %	Saturation limit γ %
1.	M	42	95	4.7	61	59	312
2.	M	34	108	4.8	17	130	294
3.	M	33	104	4.5	45	105	287
4.	M	55	95	4.8	51	42	274
5.	M	45	116	5.4	36	27	264
6.	M	35	120	5.0	15	140	258
7.	M	54	110	4.6	36	102	228
8.	M	29	89	4.1	81	61	228
9.	M	52	110	4.7	61	32	222
10.	M	68	96	4.0	72	58	216
11.	M	46	110	4.9	78	71	211
12.	M	39	60	3.8	136	34	150
13.	F	47	93	4.3	56	56	378
14.	F	28	75	4.2	31	42	372
15.	F	33	87	4.8	43	54	295
16.	F	38	83	3.8	40	144	292
17.	F	36	80	4.0	40	49	292
18.	F	54	98	4.6	66	60	288
19.	F	41	95	4.3	28	46	286
20.	F	41	90	4.4	30	86	254
21.	F	51	95	4.2	18	66	251
22.	F	26	95	4.2	53	45	248
23.	F	59	108	4.5	14	136	245
24.	F	53	90	4.3	56	73	242
25.	F	45	100	4.5	13	77	232
26.	F	56	75	3.7	70	54	230
27.	F	24	88	3.9	95	57	206
28.	F	52	96	5.0	41	59	205
29.	F	46	88	4.4	39	74	203
30.	F	59	85	4.3	88	113	198

Table 43 shows the findings in afebrile patients with *chronic polyarthritis*, and some febrile cases of the same disease are entered in Table 44.

Generally the values for serum iron obtained in the afebrile patients ($M=72 \gamma$ %) are somewhat lower than normally. In 52 cases of chronic polyarthritis, Lövgren (1945) found a markedly low mean value ($39 \pm 3 \gamma$ %). Most investigators, however, have found such low values only in febrile patients.

The mean value for the saturation limit in the afebrile patients ($255 \pm 9 \gamma$ %) is significantly lower than the mean value

Table 44.

Saturation limit in chronic rheumatic polyarthritis with fever.

Case No.	Sex	Age	Hb %	R.b.c. mill./cmm	Sedimentation rate mm/hour	Serum iron γ %	Saturation limit γ %	Temperature
1.	M	38	98	4.5	65	75	174	38
						86	257	37
2.	M	23	62	3.4	123	42	157	38.5
						83	183	37.2
3.	M	21	106	4.5	104	35	205	37

1. Repeated rise in temperature after injections of coli vaccine. Temperature falling at first sampling of blood.

2. 5 days before the first blood sampling the patient received an injection of coli vaccine.

3. 3 days before the blood sampling, rise of temperature (39°) after injection of coli vaccine.

found in normal subjects ($315 \pm 3.3 \gamma$ %). In general only the febrile patients with chronic polyarthritis and the afebrile patients in whom the sedimentation rate exceeds 70 mm./1 h. show such low values for the saturation limit as are found in acute infections.

The frequency of anemia in this material is low. Still, patient No. 12 was clearly anemic, and he also presented the lowest value for the saturation limit (150γ %).

The 20 cases of *pulmonary tuberculosis* examined in this respect are recorded in Table 45. Bröchner-Mortensen & Stein (1942) determined the serum iron concentration in 60 cases of pulmonary tuberculosis and found as a general rule that serum iron was low when the sedimentation rate was high, normal when the sedimentation rate was low.

From Table 45 it will be noticed that in the present material, which chiefly was made up by severe cases of pulmonary tuberculosis, all the patients gave relatively low values for serum iron. The values for the saturation limit show a wide dispersion, and in a majority of these cases the values fall within the limit of normal variation. Still, the mean value ($249 \pm 8 \gamma$ %) is significantly lower than the mean value for the normal material. It is a striking feature that the patients showing subnormal values

Table 45.
Saturation limit in pulmonary tuberculosis.

Case No.	Sex	Age	Sedimentation rate mm/hour	Temperature	Serum iron γ %	Saturation limit γ %
1.	F	30	22	37.1	52	326
2.	F	47	36	37.0	64	315
3.	M	16	25	37.1	49	307
4.	M	48	72	37.9	39	303
5.	F	24	70	37.6	33	290
6.	F	29	4	37.6	63	289
7.	M	26	20	38.6	51	269
8.	M	34	88	37.9	39	259
9.	F	49	68	37.4	42	248
10.	F	66		37.0	60	246
11.	M	28	56	37.2	56	234
12.	F	49	76	37.4	26	233
13.	F	28	45	38.0	26	232
14.	M		70	38.1	19	230
15.	M	31	92	38.0	49	216
16.	M	38	84	37.8	48	214
17.	F	62	60	39.0	34	212
18.	F	24	34	38.4	44	212
19.	F	53	75	38.4	31	177
20.	F	61	120	37.4	59	166
Mean value:					44	249

1. Recent process on left side, with suspect miliary spreading.
2. Recent density in left upper lobe with suspect dissolution.
3. Large progressive parenchymatous process on left side.
4. Old process. No definite progression. Still, subfebrile during the last months.
5. Accidental progression of old tuberculosis, now decreasing.
6. Increasing spreading of bilateral tuberculosis.
7. Large recent parenchymatous process in one lung.
8. Parenchymatous process in regression. Tuberculous mastoiditis.
9. Old bilateral tuberculosis in progress.
10. Bilateral tuberculosis, with chronic nephritis.
11. Bilateral processes in regression.
12. Stationary process in right lung.
13. Old stationary bilateral tuberculosis.
14. Old bilateral tuberculosis, progressing.
15. Extensive bilateral tuberculosis with cavitation.
16. Markedly fibrous process, progressing slowly.
17. Bilateral processes in roentgenological progression.
18. Tuberculous pneumonia in slow regression.
19. Old tuberculosis with bronchiectasis.
20. Old changes in both lungs. Remittent feber.

for the saturation limit generally had a subfebrile or febrile temperature — in contrast to the patients showing a normal saturation limit. No other definite connection could be made out between the clinical condition of these patients and the respective values for the saturation limit. Thus, an afebrile patient with severe progressive tuberculosis may give a normal value for the saturation limit.

Conclusion.

High-febrile, protracted infections are associated regularly with the development of anemia at the same time as the values for serum iron and the saturation limit are lowered. In contrast hereto, the appearance of anemia is relatively rare in chronic infections with normal temperature, although the values for serum iron and saturation limit are somewhat lower than normally.

Studies on the genesis of infection anemias have made it rather probable that the inhibition of the erythropoiesis in infections is not due primarily to a deficiency of iron in the bone marrow. The serum iron level is low, however, in anemia from iron deficiency as well as in anemia accompanying infection. *In iron deficiency anemia the values for the saturation limit are found to be high, whereas they are low in infection anemia. This difference may perhaps be taken to corroborate the view that the infection anemias are not due primarily to iron deficiency.*

. In infections the decrease in serum iron appears to be due to the circumstance that the liver and spleen take up more iron from the plasma than normally.

The decrease of the saturation limit in infections can depend upon the same factor as the decrease in the serum iron or possibly upon an impaired formation of the iron-binding component through the influence of the infection.

Uremic Conditions.

In chronic nephritis with increasing renal insufficiency normo-chromic anemia usually develops. As a rule, the more severe the renal insufficiency, the more pronounced is the anemia. This

Table 46.
Saturation limit in uremia.

Case No.	Sex	Age	Hb %	R.b.c. mill./cmm	Non protein nitrogen mg %	Sedimentation rate mm/hour	Serum iron γ %	Saturation limit γ %	Temperature
1.	M	63	34	2.4	100—200	145	42	186	37
2.	M	55	48	2.5	50	80	32	234	37
3.	M	69	82	4.2	110	103	16	198	37
4.	M	52	41	2.2	178	139	72	130	37
One month later			32	1.7	143	115	73	123	
5.	M	61	64	3.6	150—200	26	91	229	37
6.	F	57	49	2.8	180	59	26	176	37
7.	M	23	80	4.0	Date	116			
					16/2		10	88	39.1
					19/2		62	96	37.2
					23/2		64	115	37

1. Chronic pyelonephritis with uremia.
2. Chronic nephritis. Urea clearance about 30 %.
3. Chronic glomerulonephritis.
4. Chronic glomerulonephritis with uremia.
5. Chronic nephritis with uremia.
6. Bilateral cystic degeneration of the kidney with uremia.
7. Nephrosis; acute bronchopneumonia.

form of anemia is believed to be due to depression of the blood marrow function (for literature, see Andereggen 1946).

In chronic nephritis and uremia serum iron is usually decreased, though sometimes normal (Skouge 1939, Walker 1939, Waldenström 1940, Vahlquist 1941 and Brøchner-Mortensen 1943). In a material of 27 patients, Brøchner-Mortensen (1943) has shown that in chronic nephritis there usually is no correlation between the values for serum iron and hemoglobin.

The writer has determined the serum iron concentration and saturation limit on 6 patients with pronounced renal insufficiency (Table 46). They all showed *subnormal or low values for serum iron, besides a distinct decrease in the saturation limit.*

The low iron-binding component content of the serum may be due either to a decrease in its production or to increased loss of this component. The extremely low value for the saturation

limit observed in Case 7 (nephrotic patient with very pronounced albuminuria) suggests that the loss of albumin with the urine may contribute to a lower amount of iron-binding component in the serum.

Malignant Tumors.

In patients with malignant tumors the value for serum iron is usually low (for literature, see Brøchner-Mortensen 1943), regardless of whether any anemia has developed.

Heilmeyer (1937) holds that pathophysiologically the anemia associated with malignant tumors is closely related to infection anemias. He thinks that iron deficiency appears in the bone marrow because other tissues in the organism assimilate an increased amount of iron while the tumors are developing. Nowadays, however, the infection anemias are primarily attributed to a toxic inhibition of the bone marrow. Very likely the same applies to cancerous anemia even though iron deficiency here may be a contributory factor. Naturally, iron deficiency may readily arise in cases where the malignant neoplasms are accompanied by hemorrhage — *e.g.*, gastro-intestinal and uterine tumors. Thus Waldenström (1941) has shown that anemia in cancer of the stomach sometimes responds very favourably to iron therapy.

In order to see how the saturation limit is affected in patients with malignant tumors, a number of such patients were examined. The results are reported in Table 47. In all these patients the lesion had reached an advanced stage, and anemia had developed in most of them. In all the cases with exception of No. 3 the values for serum iron were found to be low.

As in infection anemia, the values for the saturation limit are here seen to be lower than normally, and lowest in the patients who also had fever (tumor+infection). It is to be noted, however, that in Case 12 (reticulum cell sarcoma) in which there was a temperature of 38—39°, the value for the saturation limit was still perfectly normal.

All the patients except No. 1 (cancer of the stomach) showed a normal color index. Plainly, in Case 1 there was also a con-

Table 47.
Saturation limit in malignant tumors.

Case No	Sex	Age	Hb %	R. b. c. mill./cmm	Sedimentation rate mm/hour	Temperature	Serum iron γ %	Saturation limit γ %
1.	M	83	34	2.6	53	37	20	250
2.	F	73	79	4.4	30	37	48	228
3.	M	58	85	3.7	33	37	120	240
4.	M	74	100	4.5	67	37	53	206
5.	M	62	93	5.0	14	37	46	268
6.	F	33	56	2.7	45	37	32	230
7.	F	56	44	2.6	42	37	16	146
8.	F	68	64	3.1	50	37	40	130
9.	F	60	72	3.5	19	38	21	170
10.	F	33	84	4.2	34	37.6	21	190
11.	F	77	57	3.3	75	38.4	33	216
12.	F	50	52	2.4	69	38—39	36	292

1. Cancer of stomach. 79 % reticulocytes on Fe therapy.
2. Cancer of stomach with metastasis. Ascites.
3. Cancer of bronchus.
4. Metastatic cancer (liver, skin and ribs).
5. Cancer of hypopharynx with metastasis to cervical lymph nodes, right.
6. Cancer of colli uteri. Delivery 8 months earlier.
7. Cancer of the cervix. The day before temperature was 39°.
8. Sarcoma of tonsil with metastasis to cervical lymph nodes. X-ray treatment. Anemia developing in spite of Fe therapy.
9. Cancer of ovary, inoperable. X-ray treatment.
10. Cystocarcinoma of ovary.
11. Cancer of stomach.
12. Sarcoma reticulosum.

comitant sideropenia, as the patient responded to iron therapy with a rise in reticulocytes. In Table 36, covering anemia in chronic hemorrhage, also 2 cases of cancer with melena are included. In these 2 patients the anemia was clearly hypochromic, and here the saturation limit was increased just as in other cases of sideropenic anemia. This may perhaps be taken to indicate that also patients with malignant tumors are able to react with an increase in the saturation limit when the anemia is due to real iron deficiency. Very likely the prerequisite of this normal reaction must be that the general condition of the patient is relatively unaffected by the presence of the tumor.

In patients with extensive malignant tumors the values obtained for serum iron as well as for the saturation limit are generally low — just as in infections.

Leukemias, Myelomatosis and some Other Diseases.

Patients with *myeloid leukemia* are reported to show normal or rather high values for serum iron.

This is quite in harmony with the values found by the writer in 9 patients suffering from myeloid leukemia (Table 48). Two of these patients (Nos. 6 and 8) showed normal values for the saturation limit, while the remaining 7 patients showed low values.

Table 48.
Saturation limit in leukemias.

Case No.	Sex	Age	Date	Hb %	R.b.c. mill./cmm	White cells/cmm	Serum iron γ %	Saturation limit γ %	Temperature
1.	M	39	15/11	62	3.6	3 000	69	190	38—39
2.	F	41	23/3	70	3.4	1 500	184	200	37.6
			10/4	43	2.0	3 100	91	151	38.4
3.	M	61	28/3	79	3.8	57 200	140	195	37.7
4.	M	59	31/10	57	2.7	15 000	181	263	37
			17/12	56	2.3	25 000	149	260	37
5.	F	71		65	3.2	170 000	105	232	38
6.	F	32	21/11	68	2.9	220 000	123	318	37
			10/12	70	3.7	205 000	120	300	37
			8/1	82	3.9	140 000	98	300	37
			8/4	77	4.3	22 800	37	330	37
7.	M	44	8/11	25	1.2	118 000	68	153	38
			20/11	36	1.6	24 700	151	211	37
			3/12	52	2.3	14 500	53	136	39.6
			12/12	44	2.1	80 000	65	156	37.8
8.	F	42	5/3	49	2.6	5 000	308	351	37.5
			18/3	35	1.6	2 000	250	347	38
			21/3	23	1.3	1 000	169	321	40
9.	M	30		69	3.8	95 000	71	248	37

1. Aleukemic leukemia. Fever at home since 10/10. Exitus on 17/12. 25—40 % myeloblasts.

2. Aleukemic leukemia. Acute onset on 10/3. 20—60 % myeloblasts. Blood in feces.

3. Acute myeloid leukemia. Acute onset on 8/3. 98 % myeloblasts.

4. Chronic myeloid leukemia. Tired since July. Treated with urethane.

5. Chronic myeloid leukemia. Exitus 1 week later.

6. Chronic myeloid leukemia. Successfully treated with urethane.

7. Chronic myeloid leukemia. Pulmonary tuberculosis. First appearance in May. Treated with blood transfusions and urethane.

8. Acute myeloblastic leukemia. 10 % neutrophile cells. 40—60 % myeloblasts. Exitus on 24/3.

9. Chronic myeloid leukemia. Ill over 2 years. Treated with urethane.

In Case 6 urethane therapy had a favorable effect. Under this treatment the anemia was improving at the same time as the values for serum iron were decreasing. On the other hand, the saturation limit was not increased by the treatment.

In Case 8 the disease took a fulminant course with gangrenous angina, and here a severe degree of anemia developed rapidly. At the first analysis the serum iron level was high, but subsequently it fell to a normal level. In spite of the high fever there was no change in the saturation limit, in comparison to the usual findings in acute infections.

Four patients with *myelomatosis* (Table 49) showed somewhat low values for the saturation limit and practically normal values for serum iron.

Table 49.

Saturation limit in myelomatosis, ulcerative colitis and other diseases.

Cases No.	Sex	Age	R. b. c. mill./cmm	Hb %	Sedimentation rate mm/hour	Serum iron γ %	Saturation limit γ %
1.	F	63	3.6	68	105	87	238
2.	F	57	3.1	68	50	76	232
3.	F	56	2.0	43	135	91	230
4.	F	50	3.7	73	140	100	255
5.	F	64	4.2	74	7	30	230
6.	M	24	2.7	42	19	13	274
7.	F	49	4.2	73	49	38	326
8.	F	51	3.7	45	34	22	270
9.	F	21	5.1	97	8	98	306
10.	M	18	4.6	71	14	50	300
11.	F	64	3.7	71	25	85	208
12.	M	50	1.8	34	25	70	265
13.	F	66	2.5	56	120	85	190
14.	F	14	6.2	135	1	217	433

1. Myelomatosis. Ill for 3 months.
2. Myelomatosis, diagnosed 3 years ago.
3. Myelomatosis, diagnosed $2\frac{1}{2}$ years ago.
4. Myelomatosis, established 1 year ago.
5. Lymphogranulomatosis maligna. Hepatosplenomegaly. Ill for 3 months.
6. Ulcerative colitis. Blood in feces.
7. Ulcerative colitis.
8. Chronic colitis. Blood in feces.
9. Ulcerative colitis.
10. Ulcerative colitis. Anemia in regression after Fe therapy.
11. Grafe's disease.
12. Osteomyelosclerosis.
13. Aplastic anemia.
14. Polycythaemia rubra vera.

These findings indicate — as is generally assumed — that in leukemia and myelomatosis the anemias are not due to iron deficiency.

Table 49 further shows the values obtained for serum iron and saturation limit in 5 patients suffering from *ulcerative colitis*. Four of these patients are anemic, with low serum iron. But, in contrast to the usual findings in chronic infections, the saturation limit is normal in all of them. The anemia associated with ulcerative colitis is different also in other respects — for instance, it improves under treatment with iron, in contrast to the infection anemias.

Chapter VIII.

General Views of the Intermediary Iron Metabolism.

Iron-binding Component in Serum — Apoferritin.

The iron-binding component is the protein vehicle for the transport of iron, and apoferritin is the storage protein of the organism for iron. As hitherto only apoferritin has been isolated in pure form, it cannot simply be taken for granted that the two substances are not identical.

Granick (1943) prepared an antibody against the protein apoferritin (it reacted equally well with ferritin and apoferritin). With a very sensitive precipitin test he then was able to get an idea about the apoferritin-ferritin content of various organs. He claimed that no ferritin is found in horse blood. This is in conflict, however, with the observations reported by Agner (1943): that the red blood cells of horse contain ferritin (on purification of catalase from red blood cells Agner obtained a ferritin-containing fraction). In the literature, so far, no statement is made about ferritin being present in the serum.

On comparison of the properties of ferritin (isolated in pure form according to Granick) and those of the iron-binding component in serum, some evidence has been found to indicate that the two substances are not identical.

I. When simple iron salts are added to a solution of apoferritin, no ferritin is formed. Very likely, conversion of apoferritin to ferritin requires the presence of one or more obscure components (Granick 1945). In contrast hereto, iron-binding component combines with ferrous or ferric iron added to the serum. Also when the iron-binding component is purified to a considerable extent, it reacts with iron in the same way as prior to the purification.

II. When a neutral ferritin solution is mixed with serum of high latent iron-binding capacity it has not been practicable to ascertain any conversion of ferritin iron to serum iron. If, on the other hand, a reducing substance (*e.g.*, sodium hydrosulphite) is added to the mixture, the serum undergoes a

slow change in color, getting an admixture of red — just as when simple iron salts are added. Evidently the iron-binding component takes up iron ions from ferritin in the presence of a reducing substance. If hydrosulphite and phenanthroline are added to a solution of ferritin (pH 7—8), some phenanthroline iron is found to be formed. Thus ferritin looses its iron in neutral or slightly alkaline milieu more readily than does the serum iron-protein complex.

III. When an ordinary serum iron determination is made on a ferritin solution the value obtained will amount only to a fraction of the value found on iron determination carried out on the same ferritin solution by means of incineration. This means that the iron is liberated but very incompletely from the ferritin molecules, when these are precipitated with 6 N HCl. If, on the other hand, a ferritin solution containing phenanthroline and hydrosulphite is acidified cautiously to pH about 5, most of the ferritin iron is transformed into phenanthroline iron. But in serum the iron is liberated from its protein complex at least just as well on strong acidification with 6 N HCl as on cautious acidification in the presence of phenanthroline and hydrosulphite.

These findings are interpreted as signifying that the iron is bound differently in the ferritin and in the serum iron-protein complex.

Relation between the Iron Absorption, Iron Storage and the Quotient $\frac{\text{Manifest}}{\text{Latent}}$ Iron-binding Capacity of Serum.

From the clinical material it is evident that the serum iron and saturation limit vary in an apparently regular manner in the various pathological and physiological conditions examined. Naturally, then, the question suggests itself whether there might be any connection between these variations and the storage-iron exchange.¹ There is a clear connection between ferritin and serum iron insofar as the iron is transported both to and from the depots in the form of serum iron.

From experiments reported by Hahn and collaborators we know that the iron which is liberated by destruction of red blood

¹ In this connection storage iron will have to be dealt with as if it were equivalent to ferritin, as the latter normally makes up the greatest storage-iron fraction, and besides it is the only fraction of which we have a fairly thorough knowledge.

cells under normal conditions soon is taken up again in new formed red cells. In these cases, then, the iron moves about in the circulation: hemoglobin — serum iron — hemoglobin. Normally the storage iron is practically not utilized at all, but when the regeneration of the red blood cells for some reason or other becomes more rapid than the destruction of red cells, iron is mobilized from the depots; and if the destruction of erythrocytes proceeds more rapidly than their regeneration, a part of the excess iron is transformed to ferritin.

Relation between Manifest and Latent Iron-binding Capacity in Conditions with Increased Storage of Iron.

In the following conditions, we know, that the storage-iron fraction of the organism is increasing:

- a) after intravenous injection of iron;
- b) in progressing acute and hemolytic anemias; and
- c) in the fetus, especially during the last months of pregnancy.

In these conditions the manifest and latent iron-binding capacity varies regularly:

a) After intravenous injection of iron, the value for serum iron is high, and the saturation limit normal, that is, the latent iron-binding capacity is low.

b) In acute or chronic hemolytic anemias, the serum iron is relatively high, the saturation limit normal or lowered, that is, the latent iron-binding capacity is low.

c) In the fetus the value for serum iron is normal or slightly increased, and the saturation limit is lowered, that is, the latent iron-binding capacity is low.

A characteristic feature of these conditions with storage of ferritin in the depots is that the quotient between manifest and latent iron-binding capacity of the serum is considerably higher than normally.

Relation between Manifest and Latent Iron-binding Capacity in Conditions with Mobilization of the Iron from the Depots.

In the following conditions, we know, that the storage iron fraction of the organism is decreasing:

a) after a relatively large loss of blood from acute or chronic hemorrhage;

b) in regressing acute and chronic hemolytic anemias; and

c) in pregnancy, unless the diet contains large amounts of iron.

Also in these conditions the manifest and latent iron-binding capacity of the serum is subject to regular variation:

a) After acute hemorrhages the serum iron value falls to a low level, while the saturation limit rises from a relatively low value to a relatively high level, that is the iron-binding capacity increases. In anemias from chronic hemorrhage the serum iron lies at a low level whereas the saturation limit is somewhat increased, that is: the latent iron-binding capacity is high.

b) In regressing hemolytic anemias the serum iron level is low, the saturation limit relatively low but rising, that is: the latent iron-binding capacity increases.

c) During the last months of pregnancy the serum iron level is somewhat low or normal, while the saturation limit is strongly increased, that is: the latent iron-binding capacity is greatly increased.

It is characteristic of these conditions with mobilization of storage iron that the quotient between manifest and latent iron-binding capacity of the serum is considerably lower than normally.

In the conditions where iron is mobilized from the depots, iron is absorbed more readily from the intestinal canal than normally. On the other hand, it appears — though it has not yet been established conclusively — as if the iron absorption by way of the intestinal mucosa is lesser than normally in the above-mentioned conditions where the storage-iron fraction is increasing.

On recapitulation, it may be established that the quotient between the manifest and latent iron-binding capacity of the serum is lower (0.1—0.3) than normally (0.3—1.5) as the organism has an increased requirement of physiologically active iron. Thus iron is mobilized from the depots, and the absorption of iron by way of the intestinal mucosa takes place more readily than normally. Conversely, in conditions where iron accumulates in the depots, the intestinal iron absorption will be lesser than normally.

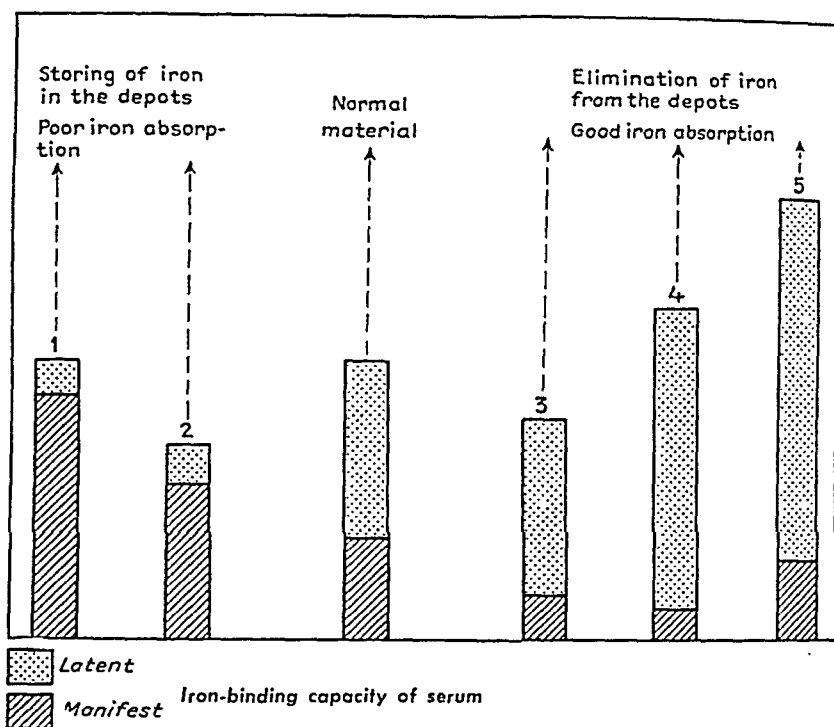


Fig. 8. Relation between manifest and latent iron-binding capacity of serum in various disturbances of the iron metabolism.

1. After intravenous injection of iron into normal subjects.
2. In acute and chronic hemolytic anemias, and in fetus.
3. In hemolytic anemia in regeneration.
4. In chronic hemorrhagic anemia.
5. During the last months of pregnancy.

As the quotient $\frac{\text{manifest}}{\text{latent}}$ iron-binding capacity of the serum plainly varies parallel with the changes in the intermediary iron metabolism, it seems rather reasonable to assume that the iron-binding component of serum serves as a balancing regulator for the exchange of iron between various organs of the body.

If the reaction $\text{Fe}^{++(+)} + \text{Protein} \rightleftharpoons \text{Fe-Protein}$ is reversible in vivo, the corresponding equilibratory reaction will be

$$\frac{[\text{Fe-Protein}]}{[\text{Fe}^{++(+)}] [\text{Protein}]} = K,$$

in which $[\text{Fe}^{++(+)}]$ signifies the activity of the iron ions in the serum.

[Protein]=iron-free iron-binding protein component of the serum.

[Fe-Protein]=iron-containing iron-binding component of serum.

In this way the serum should be able to maintain a balance of the activity of the iron ions in the body cells that are permeable to iron ions. If the activity of the iron ions decreases in one organ, iron is mobilized immediately from the depots. This is in harmony with the observations made by American investigators: that iron is mobilized from the depots (ferritin) when there is an increased demand for iron in the bone marrow, while iron at the same time is absorbed more rapidly than normal. (Granick [1946] has shown that the iron absorption is associated with a pronounced ferritin formation in the intestinal mucosa.) Indeed it also seems reasonable that in conditions where ferritin iron is mobilized from the depots, this compound is also mobilized from the intestinal mucosa at a higher rate than normally. This hypothesis implies that by lowering the quotient $\frac{\text{manifest}}{\text{latent}}$ iron-binding capacity of serum the organism is able to mobilize iron from the depots and, at the same time, improve the conditions for the absorption of iron from the intestinal tract. Correspondingly, by increasing this quotient the organism should also be able to transfer more iron to the depots than under normal conditions.

Changes in the quotient of $\frac{\text{manifest}}{\text{latent}}$ iron-binding capacity of serum might be brought about through changes in the serum iron concentration or through changes in the concentration of iron-binding component in the serum.

The prerequisite of this hypothesis is that the reaction $\text{Fe}^{++}(+) + \text{Protein} \rightleftharpoons \text{Fe-Protein}$ is reversible in vivo.

That this reaction is reversible in vitro has been shown in Chapter II, p. 53. That the reaction in vivo may proceed from left to right is evident from the iron tolerance tests reported in Chapter IV, p. 66. It has been more difficult in vivo to establish that the reaction also proceeds from right to left. The experimental and clinical data that lend support to this view are that rapid changes in the serum iron concentration may take place

in the organism without affecting the saturation limit. Thus it could be demonstrated (Chapter IV, p. 66) that the saturation limit remains unchanged when serum iron after administration of a large dose of iron falls from a high to a normal level within 12—24 hours. On treatment of pernicious-anemic patients with an adequate liver preparation, the serum iron level falls within two days from high to low values without any particular variations in the saturation limit. Therefore, it seems most likely that the above-mentioned reaction is reversible also *in vivo*.

This hypothesis is entirely schematic. Undoubtedly, the balance of the reaction is far more complicated than suggested above. Thus it may be mentioned that the iron-binding molecule is able to take up at least 2 iron atoms before it is saturated with iron.¹

This implies, among other things, that the quotient of $\frac{\text{manifest}}{\text{latent}}$ iron-binding capacity of serum in an equilibrial calculation will have to be replaced with the quotient $\frac{\text{iron-saturated}}{\text{iron-free}}$ iron-binding component. As yet, however, it has not been practicable to determine this quotient. Besides, the iron exchange between the serum and the cells of the body is further complicated by the circumstance that redox potentials and pH values vary in the various cell systems.

A general conclusion of the present work will then be that it is of no use to discuss the intermediary iron metabolism without taking the iron-binding component content of the serum into account.

¹ Studies by Laurell not published yet.

Summary.

Serum iron (the protein-bound, acid-soluble iron in the serum) is generally looked upon as the transport iron of the body. After Holmberg and the writer had been able to demonstrate that blood serum contains a specific iron-binding component, which is normally about half-saturated with iron, the work of which the results are here presented was carried out in order to investigate the significance of this serum component for transport and metabolism of iron. The starting-point for the present studies therefore was that serum contains a protein component¹ capable of binding iron (supplied in vivo or in vitro) in the same way as it is bound in the native serum iron-protein complex.

In the discussion of the experimental results obtained the term saturation limit has been introduced. The *saturation limit* of serum for iron signifies the capacity of serum for firm complex binding of iron ($\gamma/100$ ml.), and includes both the native iron content of the serum (serum iron, manifest iron-binding capacity of serum) and the increase in the concentration of firmly bound iron in the serum (latent iron-binding capacity of the serum) resulting from addition of iron to the serum.

Firstly, an account is given of the methods that have been worked out for the determination of the saturation limit.

Secondly, the results of some experiments in the binding of iron added to the serum have been described. In this way it has been possible to show:

- 1) that serum binds this iron up to the saturation limit in such a form that the iron is not adsorbed on aluminium hydroxide,

¹ Since the conclusion of the experiments here described, the iron-binding component of the serum has been isolated. Some preliminary data on this are given on p. 8.

whereas all the iron added to serum above the saturation limit is adsorbable on aluminium hydroxide. Whether the serum is enriched with a ferrous or a ferric salt, it takes up the same amount of iron in a firm complex linkage.

2) that the iron in the native serum iron-protein complex most likely occurs both in ferrous and ferric form.

3) that in a serum enriched with iron all the iron below the saturation limit is bound in a non-dialyzable form, whereas the remaining iron is dialyzable under suitable conditions.

4) that the native serum iron-protein complex begins to undergo dissociation in a noticeable degree as soon as the serum pH falls below 7.2, and not at pH 4.5 as assumed previously.

5) that the reaction iron-binding component + iron \rightleftharpoons serum iron is reversible in vitro.

Finally an account is given of some experiments that were carried out in order to ascertain the latent iron-binding capacity of the red blood cells. The results obtained suggest that this is much lower than that of serum.

The following chapters deal with the variations in the serum iron and saturation limit in normal subjects and patients suffering from various diseases.

In 100 normal subjects the mean value for the saturation limit was found to be $315 \pm 3.3 \gamma \%$.

The saturation limit was not influenced by the ingestion of a large amount of iron, even though this gave great changes in the values for serum iron.

Experiments with pregnant women showed that during normal pregnancy the values for the saturation limit began to rise regularly in the sixth month, and they kept rising till the ninth month inclusive, while they showed a tendency to fall immediately before parturition. Thus the values for the saturation limit appeared to rise parallel with the increased iron requirement in the latter part of pregnancy.

Serum analyses were also carried out on 25 mothers and children at parturition, and it was found that the serum iron level in the newborn ($M=146 \gamma \%$) was considerably higher than in the normal subjects, whereas the saturation limit ($M=226 \pm 10 \gamma \%$) was significantly lower than normally. In the

mothers the opposite was found to be the case, the serum iron values being lower than normally ($80 \gamma \%$), while the serum saturation limit values ($M=446 \pm 13 \gamma \%$) were higher than normally. The observations reported by Pommerenke, Hahn *et al.* show clearly that the fetus obtains its iron at least in part via the maternal serum iron. The differences in the values for the saturation limit and serum iron found in the mothers and children indicate that the iron-binding component of the maternal serum cannot freely pass through the placenta.

Large acute losses of blood are followed immediately by a slight fall in the saturation limit. Subsequently the value rises above the initial values within one week, at the same time as the values for serum iron decrease. In anemias from chronic hemorrhage the values for the saturation limit are higher than normally and the values for serum iron are low.

In patients with untreated pernicious anemia the values for the saturation limit were somewhat lower than normally ($M=236 \pm 9 \gamma \%$). On the institution of liver therapy the values for serum iron were found to fall rapidly in the first 48 hours, without the saturation limit being affected.

In patients with acute infections there was an abrupt fall in serum iron as well as in the saturation limit in the first days of illness. The fall in these two values ran parallel in contrast to those found in pernicious anemia under liver therapy, where there is no change in the saturation limit when the serum iron decreases. Later on, when the infection subsided, the values for the saturation limit slowly returned to a normal level. In chronic infections (tuberculosis, chronic rheumatic polyarthritis) the saturation limit was somewhat lowered but not to such an extent as in acute infections.

Something similar was apparent in patients suffering from malignant tumors, leukemia and various other diseases.

In acute hepatitis the values for the saturation limit were normal during the first days of illness, but subsequently showed a tendency to rise. This rise in the saturation limit seemed to make its appearance somewhat later than the rise in serum iron. In patients suffering from cirrhosis of the liver and from uremia the saturation limit was found to be much lower than normally.

In the last chapter the results here reported are discussed with relation to previous experimental observations on the intermediary iron metabolism.

From the experiments published by Hahn and collaborators we know that the iron which is liberated at the destruction of red blood cells is rapidly taken up again in new formed red blood cells. Thus most of this iron runs through the cycle: hemoglobin — (depot iron) — serum iron — (depot iron) — hemoglobin. Normally the depot iron is practically not utilized, but if, for some reason the regeneration of red blood cells proceeds more rapidly than the destruction of red blood cells, iron is mobilized from the depots. Conversely, if the red cells are destroyed more rapidly than they are regenerated, a part of the surplus iron is deposited as ferritin.

From the clinical material it is evident that serum iron and the saturation limit vary regularly in the various physiological and pathological conditions examined.

As established already, the depot iron fraction increases under the following conditions: after intravenous injection of iron, in acute or in chronic hemolytic progressive anemia, and in the fetus during the last months of pregnancy. One feature characteristic of these conditions, with deposition of iron in the depots is that the quotient $\frac{\text{manifest}}{\text{latent}}$ iron-binding capacity of serum is larger (>1.5) than normally. On the other hand, this quotient is smaller (<0.3) than normally in cases where the depot iron is decreasing (after considerable bleeding, chronic or acute, in acute or chronic anemias in regression, and during the last months of pregnancy). In the last mentioned conditions, iron is also absorbed more rapidly than is generally the case.

As the quotient $\frac{\text{manifest}}{\text{latent}}$ iron-binding capacity of serum appears to vary regularly with changes in the intermediary iron metabolism, it seems probable that the iron-binding component in the serum serves as a regulator for the iron exchange between the various organs of the body, and this has to be taken into consideration when the mechanism of iron absorption is discussed.

On the basis of the results obtained, a hypothesis is advanced concerning iron metabolism, of which the following are the main points:

1) that the organism is capable by lowering the quotient of manifest iron-binding capacity of the serum (either by lowering latent the serum iron concentration or by increasing the iron-free iron-binding component present in the serum) to mobilize iron from the depots and, at the same time, to improve the conditions for absorption of iron.

2) that by increasing this quotient the organism is able to transfer more iron than under normal conditions to the iron depots, and at the same time to check the absorption of iron.

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Reciprocal Effects due to Stimulation of the Spinal Cord by Currents of Opposite Direction

By C. R. SKOGLUND

Introduction

BARRON and MATTHEWS (1936, 1938) found that vertebrate motoneurons respond rhythmically to direct current stimulation. Their experiments on frogs and cats demonstrated that polarisation of the central part of the spinal motoneurons elicited regular discharges in the motor root when the cathode was placed on the cord and the anode on the root, whereas a current in the reverse direction only occasionally evoked responses.

In the course of an analysis of spinal functions in cat by polarisation of the cord and by recording the activity in different muscles, SKOGLUND (1946) observed that currents of a given direction caused activity in flexor muscles, while a reversal of the current resulted in extension.

This observation was found to be in agreement with results from experiments in which lower animals were exposed to current fields of opposite directions. In order to analyse the phenomenon of galvanotropism, described by HERMANN (1885), LOEB and MAXWELL (1896) sent galvanic currents through the water in a trough containing shrimps (*Palaemonetes*) and found that the motion of these animals to the anode is caused by characteristic changes in tension of associated muscles. When the animal is placed with its head towards the cathode, the tail is bent ventrally, which means that the tension of the flexors is stronger than that of the extensors

and at the same time the fifth pair of legs is also bent, while the third pair of legs is extended. When the current is reversed, the tail and the fifth pair of legs are stretched, while the third pair is bent. Other crustaceans, like the common crayfish, as well as larvae of salamander showed the same reaction (LOEB and GARREY 1896).

In modern studies of central nervous functions, very little attention has been paid to these phenomena of selective activation of reciprocal systems by currents of opposite direction, described also in LOEB's monograph (1918). The only contribution, as far as I am aware, is made by PRINGLE (personal communication) who made the interesting observation (1946) that application of currents of opposite direction to the ventral motor ganglia of insects produced extensor and flexor activity of low rhythms.

The present paper gives a more detailed description than my previous report of experiments in which antagonistic effects are evoked by stimulating the lumbar cord of the cat with currents of different directions.

Methods

The stimulator, previously described in detail (SKOGLUND 1942), is fitted with an out-put valve with high internal resistance, in order to minimize the effect of variations in the resistance of the tissues. The stimulating electrodes placed on the surface of the cord were chlorided silver wires covered with cotton soaked in Ringer solution; the electrodes inserted in the cord were steel-needles insulated except at the point. The different electrode positions are described in the text.

Twenty cats were used for the experiments. The cats were decerebrated, the lumbar part of the cord was exposed and the dorsal roots cut. The effects were determined by observation of the movements of the intact leg or of contractions in individual muscles exposed by removal of the skin. Concentric needle electrodes were used for recording the activity in different muscles. A condensor-coupled amplifier was connected with each of the two beams of the cathode ray tube.

Results

Typical effects with different electrode arrangements.

The typical results obtained with different positions of the electrodes will first be described (see Fig. 1).

When both stimulating electrodes were placed on the lateral side of the cord in longitudinal direction (A—B), stimulation with a current of moderate strength caused flexion when the most cranial electrode A was negative, while a reversal of the current gave flexion of the ipsilateral hind leg. The selective effects were most pronounced when the distance between the electrodes was about 2 centimeters.

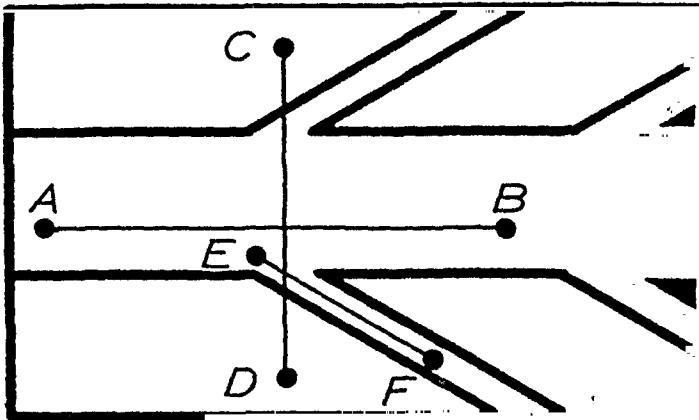


Fig. 1. Diagrams of different electrode arrangements. A—B: electrodes on the lateral surface of the cord in longitudinal direction. C—D: one electrode on each side of the cord. E—F: one electrode on the cord near the entrance of the motor root, the other on the root.

When the electrodes were placed on the dorsal muscles, one on each side of the cord so that it was exposed to a transverse flow, extension predominated on the side of the positive electrode and flexion on the side of the negative.

In another series of experiments one electrode was placed on the cord and the other on one of the motor roots (E—F). It was found that when the electrode on the cord was negative, the muscle contractions were most pronounced in the flexor muscles innervated by the root in question and that when the cord elec-

trode was positive the activity in the extensor muscles predominated.

Some experiments were also performed with one electrode on the cord and the other anywhere on the dorsal muscles. In this case too, flexor responses had the lowest threshold of stimulation when the cord electrode was negative, while extensor responses were favoured by the opposite current direction.

Although flexor and extensor movements were those most easily differentiated, adduction and abduction could also be studied. Adductor movements were usually elicited by the same current direction which caused flexor responses, while abductor movements were caused by the opposite.

When the stimuli were sufficiently strong, contralateral effects appeared which were of the same type or antagonistic to those on the ipsilateral side, apparently depending on differences in current spread.

As a rule, break of the current evoked responses in the muscles opposite to those activated during the current flow. This phenomenon was first observed accidentally during earlier experiments with artificial stimulation of the cord. Since it was obvious that the different reactions were principally due to the opposite current flow at make and break, the make responses to stimuli of opposite direction were subjected to a systematic analysis.

The reciprocal effects were more or less pronounced in different experiments. It was sometimes observed that the characteristic differences disappeared towards the end of long experiments, when the state of the cord had become deteriorated *e. g.* due to poor circulation.

Usually the stimulating current was gradually increased to threshold and suprathreshold values and when the stimulating current became sufficiently great both groups of muscles could be activated by a current of one direction. LOEB and MAXWELL (1896) pointed out that selective effects in antagonistic muscles of *Palaemonetes* were only evoked by medium strong currents, while stronger currents independent of the direction caused general rigidity of the extremities. The galvanonarcotic effects of strong direct currents on the central nervous system have been extensively studied by SCHEMINZKY (1936 a, b).

In some experiments the effect of a stimulus opposite to that causing extension was merely a relaxation of the extensor muscles, and not active flexion. A longitudinal current flow of descending direction (in Fig. 1, *A* positive and *B* negative), which generally evokes extension, may sometimes cause flexion. However, by reversal of the current the antagonistic muscle group was always activated. This reciprocal excitation by currents of opposite direction is the most important fact emerging from the analysis.

Reciprocal excitation and inhibition studied in single motor units.

Although reciprocal effects can be determined by observing muscle contractions, recording of the action potentials gives more accurate information of the activity in the respective muscles.

In the experiment illustrated in Fig. 2, one of the concentric needle electrodes was placed in *tibialis anticus* and the other in *soleus*. The extensor effect was recorded on the upper beam, the flexor activity on the lower. *A*, *B* and *C* are continuous recordings during steadily increasing stimulation with the cathode on the cord and the anode on the motor root. Typical repetitive discharges of increasing frequency appear in the flexor muscle, while no activity at all is seen in the extensor muscle, until break at the arrow in *C*.

In *D*, *E* and *F* the current is reversed and, consequently, the typical discharges now appear in the extensor muscle. The stimulus applied was stronger than in *A*, *B* and *C*, resulting in extensor discharges of a very high frequency representing a strong muscle contraction; however, there is still no activity in the flexor muscles.

Phenomena of reciprocal inhibition were frequently observed in these experiments. A current direction causing excitation of *e.g.* an extensor muscle will exert an inhibitory effect on the antagonistic flexor muscle. This is illustrated in Fig. 3, where the upper tracing gives the flexor and the lower the extensor response. The records are from an experiment in which a spontaneous activity in the flexor muscles occurred, represented by

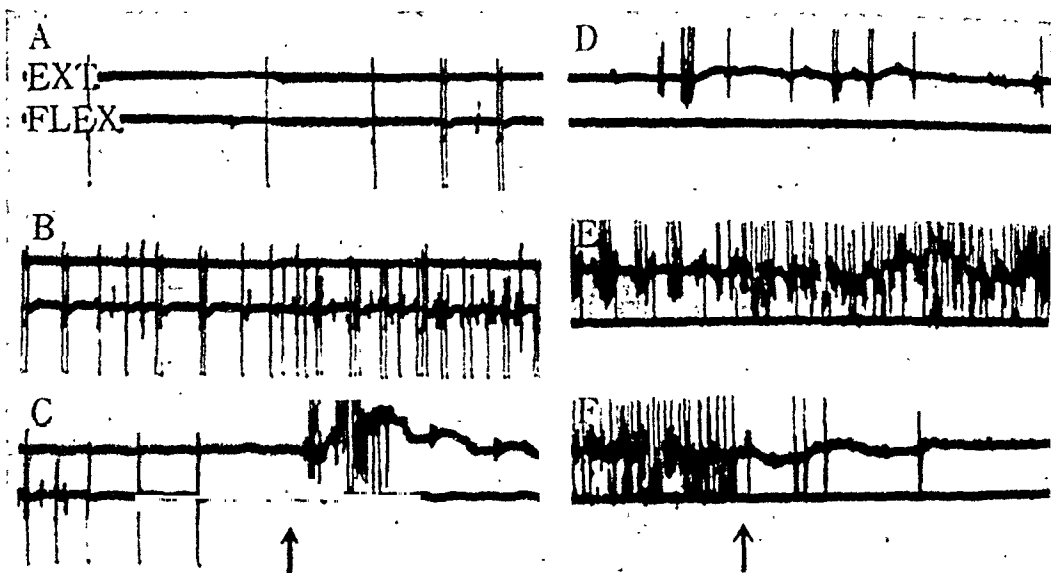


Fig. 2. Selective activation of antagonistic muscles by currents of opposite direction. Simultaneous recording of the activity in *soleus* on the upper beam (EXT.) and *tibialis anticus* on the lower beam (FLEX.). A—C: stimulation with the cathode on the cord and the anode on the root (L_7); D—F: after reversal of the current.

the discharges seen to the left in the record. The duration of the stimulus is indicated by the black line uppermost in the record. The current direction was such as to stimulate the extensor muscles and simultaneously the spontaneous activity of the flexor muscle was inhibited, only to reappear when the stimulus was removed. It is impossible to determine whether the inhibition of the flexor activity is a direct effect of the applied stimulus or a secondary effect due to the activation of the extensor system. However, in some experiments, where the stimulus strength was increased very slowly, it could be observed that inhibition of the flexor activity was blocked before any excitatory effects were recorded from the antagonist.

In the decerebrate preparations spontaneous discharges in the extensor muscles were frequently recorded and these could be accelerated by a current in one direction and inhibited by the opposite. These phenomena are similar to the facilitation and in-

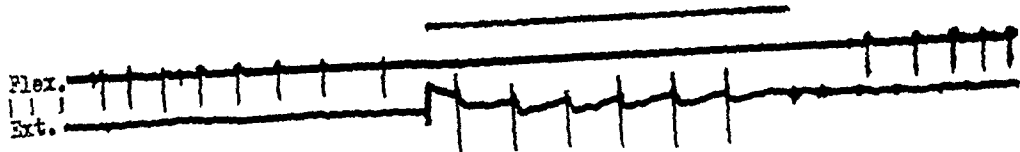


Fig. 3. Reciprocal inhibition. Simultaneous recording of flexor activity in *tibialis anticus* and extensor activity in *gastrocnemius*. Stimulation with the anode on the cord and the cathode on the root (L_7). See text.

hibition of spinal reflexes in frogs under polarisation in opposite directions described by SCHEMINZKY (1936 b).

The rising gradient of the stimulating current plays a rôle in the production of selective effects. In most of the experiments the increase of current strength was obtained manually by turning the knob of a potentiometer with the result that the rising time of the current was at least some seconds. The reciprocal effects were most pronounced with this method of stimulation but currents of shorter time constant may also be effective and differences in thresholds can be demonstrated. In one experiment with instantaneously rising currents the strengths necessary to evoke effects in *tibialis anticus* and *gastrocnemius* (using muscle potentials as index) were 12.5 and 19 μA respectively when the cathode was on the cord and the anode on the root, and 20 and 15 μA after reversal of the current.

The opposite influence on the excitability of the flexor and extensor systems caused by a current field of a given direction is demonstrated in the experiment shown in Fig. 4. Square waves were applied through one electrode on the cord (positive) and through another on the motor root (negative). The responses were recorded from the nerves to *tibialis anticus* and *gastrocnemius*. The current strength was increased until a synchronized volley appeared not only in the extensor nerve but also in the flexor nerve (a). A constant current field of the same direction as the test stimulus was then applied through the same electrodes. The effect, shown in b, is very striking, namely very nearly complete inhibition of the flexor and an increase to maximum amplitude of the extensor responses. Similar phenomena will be analysed in a later article (Skoglund 1947).

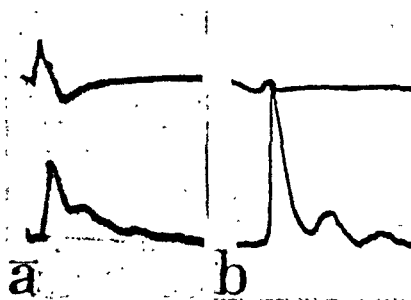


Fig. 4. Opposite influence on flexor (upper tracing) and extensor responses (lower tracing) by constant polarisation in a given direction. Full description in text.

Monopolar stimulation with inserted needle electrode.

In some experiments the current was applied via a monopolar needle electrode in the cord and an indifferent electrode placed elsewhere. When relatively strong currents were applied, reciprocal effects could be elicited from almost any point of the segment and were even more pronounced than when surface electrodes were used.

With currents of threshold strength many different points had to be tried before any selective effects were obtained. It is not the purpose of the present investigation to determine those areas from which reciprocal effects can be produced by reversal of the stimulus, but only to show that they can be localized. Stimulation with direct currents as well as instantaneously rising currents at the region indicated by a filled circle in Fig. 5 evoked selective responses in *tibialis anticus* when the needle electrode was negative while a reversal of the current caused effect only in *gastrocnemius*.

Comparison between central and peripheral stimulation of motoneurons.

The pronounced repetitive responses of central nervous structures to direct current stimulation, described by BARRON and MATTHEWS (1938), have been verified in these experiments. The duration of the centrally evoked responses to currents of different

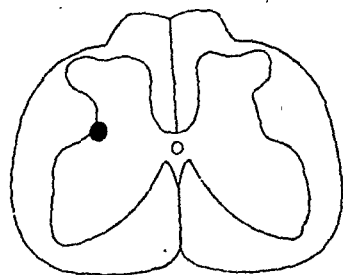


Fig. 5. Diagram of the spinal cord at the level of L₇ (see text).

strengths were measured and the result compared with those from stimulation of the peripheral part of the mononeurons. The responses of motor nerves *in situ* to direct currents of 2 and 3 times the rheobasic strength last maximally one or two seconds (SKOGLUND, 1942). The discharges evoked from central structures continue during several minutes after the onset of stimulation, as illustrated in Fig. 6. Record *a* shows the discharge of a single motor unit in *quadriceps* when a direct current of twice the rheobase is applied to the cord. The initial frequency of 20 per sec. (in *a*) has decreased after 3 seconds stimulation to 5 per sec. (in *b*), but it is still about the same after one minute (in *c*). The discharge ceases when the current is broken (*d*).

Records *e*—*f* show the long-lasting responses to a current of three times the rheobase (see Figure text). In many experiments the motor units discharge as long as the current is applied.

Low threshold values were also characteristic for central nervous tissue. A few microampères often suffice to maintain long-lasting discharges. However, when the motor root was divided and the peripheral end stimulated, the threshold was found to be 10—100 times greater.

Discussion

A different orientation of the excitable elements is the most obvious explanation of the phenomena described. LOEB and MAXWELL (1896) presented diagrams for the arrangement of neurons in the central nervous system of *Palaemonetes* necessary



Fig. 6. Long-lasting repetitive discharges (in *quadriceps*) to direct current stimulation of the cord (anode on the cord; cathode indifferent. *a*—*d* fully described in text. *e* initial response to a direct current of 3 times the rheobase (frequency about 50 per sec.); *f* after 8 seconds stimulation (20 per sec.); *g* after 12 sec. (10 per sec.); *h* after 2 minutes. Time in 20 msec.

to bring about the typical galvanotropic reactions both to sidewise and lengthwise polarisation. They assumed, partly on the basis of histological work on crustaceans, that the cell-bodies of the flexor neurons are situated on the same side as their muscles, while the extensor neurons originate from the contralateral side of the central nervous system.

The data on the localisation of flexor and extensor cell groups in the spinal cord of mammals are very incomplete. There are some indications that the ventral horn cells of the neurons to flexor and extensor muscles acting at the same joint are situated at different levels, but no definite conclusions can be drawn, especially since there are great variations in different members of the same species (see *e.g.* ELLIOTT 1942).

MARINESCO (1904), from degeneration experiments on dogs and cats, found that flexor and extensor neurons originate from different areas in the lumbar segment. ROMANES (1941) studied the embryological development of the cell columns in rabbits and

found that, initially, within any one column the cells supplying flexors and extensors of the segment innervated by that column existed side by side. Later when the cells differ functionally from each other the columns break up into several parts.

A functional method for the central localisation of different motoneurons was used by ÅSTRÖM (1947), who recorded the responses in different peripheral nerves to microstimulation of the cord. His preliminary results seem to indicate that, in the lumbar segment of the cat, the extensor neurons originate from an area dorsal to the flexor neurons.

A complete knowledge of the loci for the different cell groups in the spinal cord is required in order to determine the part played by the orientation of the elements active in reciprocal excitation.

The results can be explained in quite a different way by assuming different properties of the central elements belonging to the antagonistic systems. It is a well known fact that peripheral motor nerves have a lower threshold for descending than ascending currents but that under certain conditions a reversal of the threshold relation may occur (see *e. g.* SKOGLUND 1945). Assuming that the excitable elements in the cord have, *sui generis*, different inherent properties, so that *e. g.* the extensor neurons are most easily stimulated by ascending and the flexor neurons by descending currents, the explanation of reciprocal excitation and inhibition produced by artificial stimulation would be simple. Both processes can be imitated in model experiments on peripheral nerves containing fibres with different threshold relations for stimuli of opposite direction (*v.* EULER and SKOGLUND 1947).

It cannot be determined at the present stage whether either or both of the two factors discussed are involved in the mechanism of the reciprocal phenomena produced by artificial stimulation.

Summary

The effects of direct current stimulation of the spinal cord in cats have been studied by observing the leg movements and by recording the action potentials from different muscles and nerves.

The cord was exposed to current flow of different directions by placing the stimulating electrodes in various positions on the exposed lumbar region. With the electrode in a fixed position, stimulation with gradually increasing currents in one direction causes mainly responses in flexors, while activity in extensors predominates when the current is reversed.

Reciprocal inhibition by stimuli of opposite direction is also demonstrated.

The mechanisms of these phenomena are discussed.

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**Reciprocal Effects Evoked by Stimulation
of the Descending Motor Tracts with Currents
of Opposite Direction**

By C. R. SKOGLUND

Introduction

It was first observed by LOEB and MAXWELL (1896) in experiments on shrimps that antagonistic systems are activated when the central nervous system is exposed to currents of opposite direction and recently the phenomenon has been demonstrated on cats by applying the stimuli to the cord (SKOGLUND 1946, 1947). Similar effects produced by stimulation of the ventral motor ganglia in insects have been reported by PRINGLE (1946). The phenomenon can be explained by assuming either that the excitable elements are differently orientated in relation to the current flow or that they have different inherent excitability properties. A further analysis of the mechanisms involved in the reciprocal phenomena evoked by stimulation of the spinal cord of the cat is at present difficult owing to incomplete histological data on the cord.

The problem can, however, be differently attacked by investigating whether other parts of the motor system exhibit similar characteristics or not. In the experiments described in this paper, the stimulation has been applied to the motor tracts in the medulla and it was in fact easily demonstrated that reciprocal effects could be elicited by changing the direction of the stimulus

(cf. SKOGLUND 1946). A preliminary analysis has been made of the mechanism of this selective activation caused by medullary stimulation.

Methods

Decerebrated or Dial-anaesthetised cats were used in the experiments. The ventral side of the medulla oblongata was exposed between the base of the skull and the first vertebra. In most of the experiments laminectomy of the lumbar part of the cord was performed and the dorsal roots L_4-S_2 cut. Direct currents of various duration were applied to the medulla either through surface electrodes (chlorided silver wires of 0.5 mm. diameter covered with cotton) or through a needle electrode inserted in the medulla and an indifferent electrode placed in the soft tissues of the neck. (For description of the stimulator see SKOGLUND 1942.) The needle, insulated except for the tip, was mounted in a micrometer gauge. In order to determine the path followed by the needle electrode the formol-fixed spinal cord was frozen and serial sections studied.

The motor effects were determined by observation of the leg movements and/or by recording the effects in different muscles or nerves of the hind leg. Condenser coupled amplifiers were used.

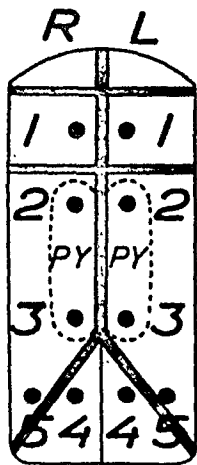
Results

Stimulation with electrodes placed on the ventral surface of the medulla.

The first explorative experiments were performed with the stimulating electrodes placed on the ventral surface of the medulla oblongata. The direct current was successively increased until motor effects in the hind leg were produced. Since the forelegs were necessarily fixed or denervated in order to prevent movements in the upper part of the body, their reaction could not be systematically studied.

Analogous to the previous experiments on the spinal cord, the pyramids were first exposed to a longitudinal current flow by

Fig. 1. The exposed ventral part of the medulla oblongata (schematic) with the vertebral arteries, the basilar artery and the pyramids (PY). Filled circles (1, 2, 3, 4 and 5): positions of stimulating electrodes on the right (R) and left (L) side. (See text.)



stimulation through two electrodes (2 and 3 in Fig. 1) placed on one of the pyramids some mm. from the midline. It was found that stimulation in one current direction (2 positive and 3 negative) produced flexion in the hind leg, while stimulation in the opposite direction caused extension.

If both electrodes were placed in the medulla at the same level, the positive on one side and the negative on the other side of the midline (*e. g.* R_2-L_2), extension on one side was simultaneously observed with flexion on the other.

With one electrode indifferent anywhere in the tissue and the other in either of the positions 1—5, flexor movements predominated when the active electrode was negative and extensor movements when it was positive.

In all cases muscle contractions were evoked on both sides. The relation between ipsi- and contralateral effects was not systematically studied. It was, however, observed that the contralateral effects were most pronounced when the active electrode was situated cranially in 1 or 2, while with the positions 3, 4 and 5 the ipsilateral effects dominated. The position of the indifferent electrode had no influence whatsoever.

Typical reciprocal effects were obtained in most experiments. In some of the decerebrate preparations, especially those in which deafferentation had been performed, the threshold of one of the systems was sometimes raised so that no effects could be evoked with currents of normal strengths. When very strong stimuli were used both systems could be activated by one current direction.

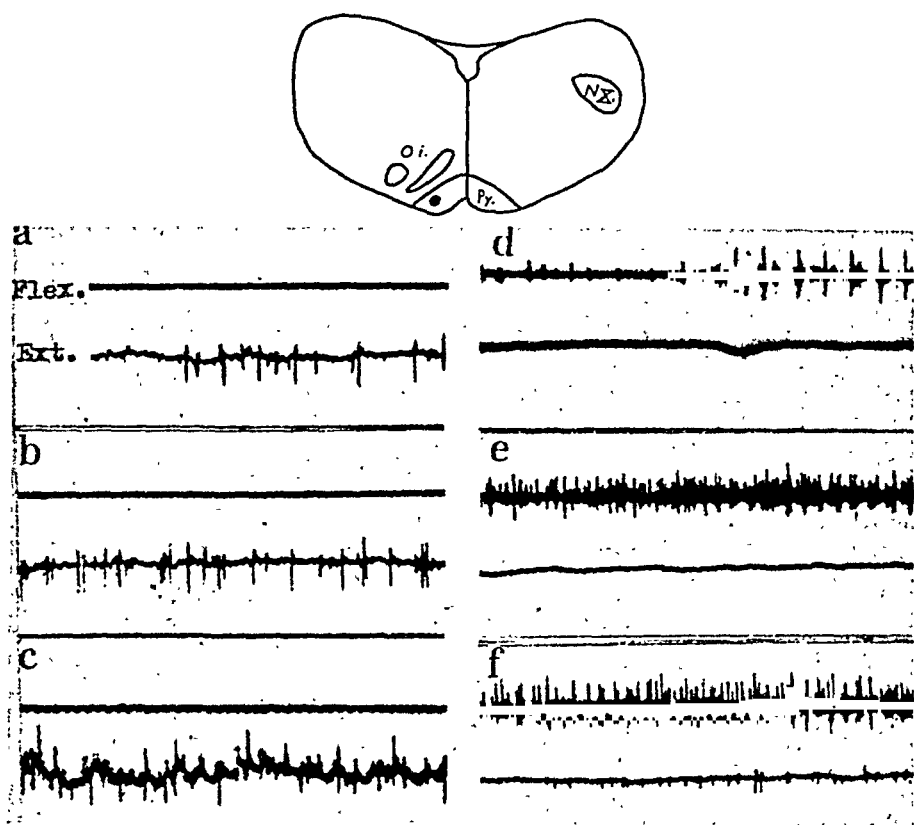


Fig. 2. Reciprocal excitation of muscles in the hind leg by stimulation of the medulla. Simultaneous recording of the activity in *biceps femoris* on the upper beam (Flex.) and *quadriceps* on the lower beam (Ext.) during stimulation (a—c) with inserted needle electrode positive and (d—e) after reversal of the current direction. Section of the medulla showing the position of the stimulating electrode (filled circle) in the right pyramidal tract (Py.). O. i. inferior olive.

Stimulation with inserted needle electrodes.

When surface electrodes were used the results from the different experiments were sometimes variable and it soon became obvious that this was partly due to current spread. Therefore the current flow was concentrated to smaller areas by insertion in the medulla of a needle electrode. Uniform antagonistic effects were easily obtained by this method. In the typical experiment shown in Fig. 2 the needle electrode was inserted in the pyramid on the right side and the effects recorded on the left side in *tibialis*

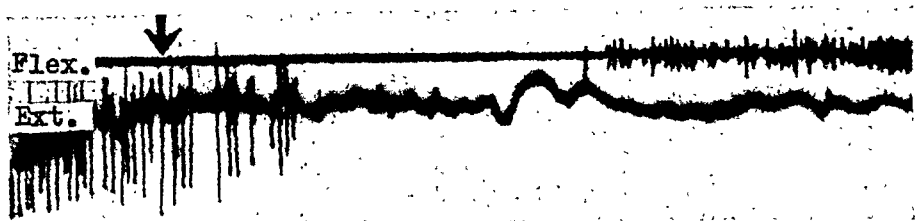


Fig. 3. Reciprocal inhibition of muscles in the hind leg by stimulation of the medulla. Simultaneous recording of activity in *tibialis anticus* (Flex.) and *gastrocnemius* (Ext.). At the arrow onset of negative stimulation. (See text.)

anticus and *gastrocnemius*. When the needle electrode was positive, direct current stimulation caused repetitive discharges in the extensor muscle, while no activity appeared in the flexor muscle (*a*, *b*, and *c*). Stimulation in the reversed direction caused the results shown in *d*—*f*. When low- and medium current strengths were used, the flexor muscle was selectively activated; not before the current reached a very high value did small discharges appear also in the extensor muscle (*f*).

Not only reciprocal excitation but also reciprocal inhibition could be produced by artificial stimulation of the medulla. It was found that the same current direction, which caused excitation of a flexor muscle also caused inhibition of an extensor muscle and vice versa. This is illustrated in Fig. 3. The animal used in the experiment had a pronounced decerebrate rigidity and therefore spontaneous extensor discharges occurred (seen to the left in the record). When the negativity of the needle inserted in the pyramid was steadily increased, the first effect was inhibition of the extensor discharges later followed by activity in the flexor muscle. It is probable that this inhibitory effect is due to blocking of impulses from higher centres passing through the medulla but the possibility of a secondary effect at lower spinal levels due to activation of descending tracts with inhibitory functions should not be excluded (cf. MAGOUN and RHINES 1946).

In the decerebrate animals clonus movements often appeared and these were influenced by direct current stimulation of the medulla in such a way that the clonus was increased by stimulation in one direction (inserted electrode positive), while it was inhibited by a current of the opposite direction.

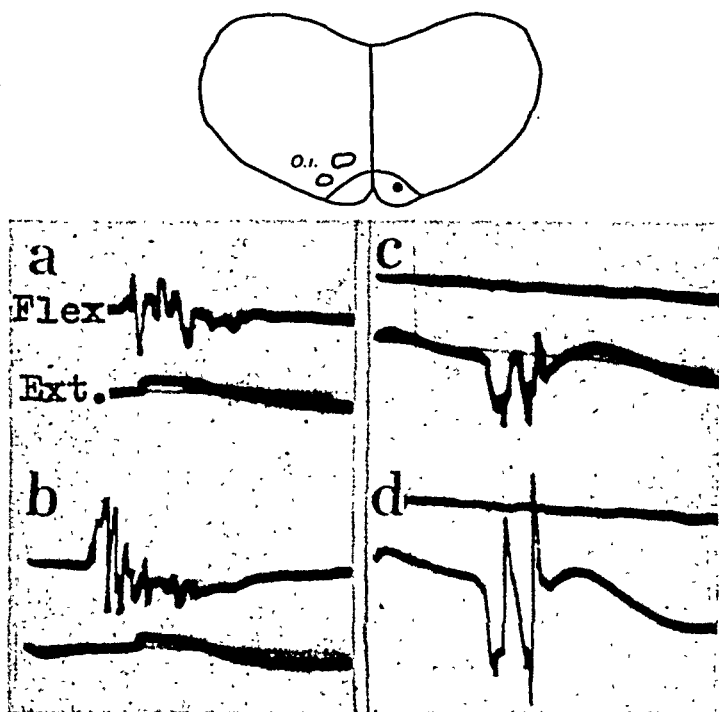


Fig. 4. Selective responses in *tibialis anticus* (Flex.) and *gastrocnemius* (Ext.) to square wave stimulation of the medulla. Stimulus duration 22 msec. *a* and *b*: negative stimulation with different strengths, *c* and *d*: after reversal of the current. Section of the medulla through the proximal end of the inferior olive (O. i.) showing the position of the stimulating electrode (filled circle) in the pyramid.

In the previous experiments the stimulus was slowly increased to threshold strength. Typical results were also obtained with instantaneously rising currents. In the experiment illustrated in Fig. 4, square waves of 20 msec. duration were applied. Record *a* shows the selective response in *tibialis anticus* to a negative threshold stimulus. Even with the maximum stimulus (in *b*) no activity at all is seen in the extensor muscle. Records *c* and *d* show the selective activation of *gastrocnemius* after reversal of the current direction. As a rule the thresholds were about the same for both effects, as appears from Table I.

In some experiments the latency of the peripheral response to square wave stimulation of the pyramidal tract was determined by recording from one of the lumbar roots. In the experiment

Table I.

Exp. No.	Relative threshold values of	
	negative currents causing flexion	positive currents causing extension
11	0,8	1,0
12	0,9	0,9
16	0,12	0,12
25	0,4	0,4
43	0,37	0,24

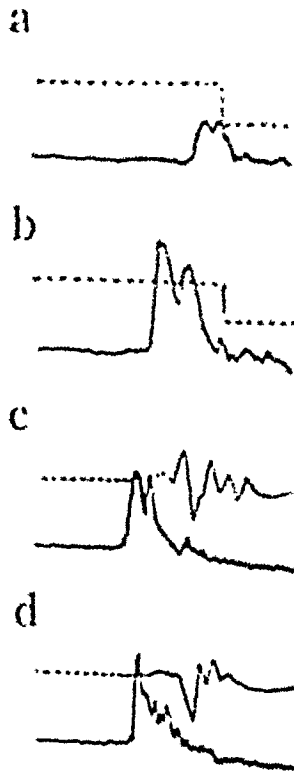


Fig. 5. *a* and *b* recordings of the discharge in the 7th lumbar root to medullary stimulation with a direct current of 15 msec. duration (marked on the upper beam. Time in msec.).

c Simultaneous recording of the response in the 7th lumbar motor root (on the lower beam) and the activity in *tibialis anticus* (on the upper beam) when a negative stimulus of 15 msec. duration is applied in the medulla.

d Same as in *c* although recording the activity in *gastrocnemius* (on the upper beam) and with positive stimulation.

shown in Fig. 5 the latency with threshold stimulation was about 12 msec. (a) and with maximum stimulation about 9 msec. (b). The lowest values observed were about 7 msec. (cf. LLOYD 1941 b). It was interesting to compare the latencies of the motor root responses when the two systems were activated by medullary stimulation. An intact motor root (L_7) was placed on the recording electrodes so that the impulse discharge in the root could be recorded at the same time as the muscle responses were recorded. In Fig. 5 c the latency of the response in the root is 9 msec. (and that of the action potential in *tibialis anticus* about 14 msec.). In d the stimulating current was reversed and a selective response was now evoked in the extensor muscle (*gastrocnemius*). The latency of the ventral root discharge is the same as when the flexor muscle was activated *i.e.* 9 msec. (The muscle action potential had a latency of 17 msec. This difference between the latencies in the two cases is due to different conduction times in the respective nerves and muscles.) The results from these observations thus show that the latencies of the motor root responses are the same when flexor and extensor effects are evoked. This indicates that the excitation and conduction processes are very similar in both systems.

Determination of the loci of excitation.

Although the density of the current flow is greatest near the tip of the needle, excitation may occur at any point within the current field provided that the threshold of the excitable elements in this area is sufficiently low. The locus of excitation can be roughly determined by observing the reactions when incisions are made cranially and caudally to the stimulating electrode. Wherever the needle is situated a complete transection cranially has no observable effect. This shows that ascending impulses reaching higher centres cannot be responsible for the production of the reciprocal phenomena. With the needle in the pyramid (as in the diagram in Fig. 3), a caudal transection of the pyramid causes, in most cases, a reduction of the response. With the needle in a deeper or more lateral position (cf. below) the effects are inhibited

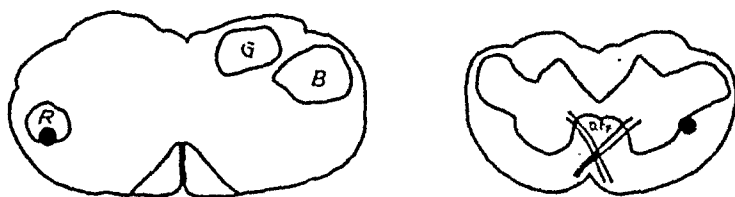


Fig. 6 a. Section of the medulla near the transition into the spinal cord. G: Nucleus Goll. B: Nucleus Burdach. R: rubrospinal tract. Filled circle: low threshold area for the elicitation of reciprocal effects.

Fig. 6 b. Section of the medulla through the middle of the pyramidal decussation (D. Py.) Filled circle: as in 6 a.

only when a transection of the lateral part of the medulla is made, thus indicating that extrapyramidal tracts are activated by the stimulating current.

In order to localise the excitable structures more precisely, the needle was inserted in different directions through the medulla; the areas of lowest threshold were determined and the depth of the needle read on the scale of the micrometer gauge. When the needle was inserted in one of the pyramids the lowest threshold for the typical effects was found 1.0—1.5 mm. from the surface. This would suggest that the excitation takes place in the pyramids when threshold stimuli are used. Pressing the needle deeper into the medulla, the effects diminished and 3—4 mm. from the surface they disappeared. However, stronger currents could restore the effects at these different depths.

Threshold determinations have also been made by inserting the needle in different lateral positions. The diagrams in Fig. 6 illustrate some typical results from these investigations. In many experiments low threshold areas were found at depths corresponding to extrapyramidal tracts (Fig. 6 a). With the needle in caudal position (Fig. 6 b) the crossed pyramidal tract may also have been involved. It should be noted that the low threshold areas were usually the same for both flexor and extensor effects.

The results primarily indicate that the descending tracts are directly stimulated but a current spread to the different cell groups in the medulla cannot be eliminated at the present stage in the investigations.

Influence of constant polarisation on the excitation processes.

When the antagonistic systems are activated by short direct current pulses, the thresholds of stimulation remain unchanged during long periods. Therefore it is possible to study the influence of constant polarisation on the excitability in the flexor and extensor systems, analogous to the classical experiments on peripheral nerves.

In the experiment described in Fig. 7, a stimulus of 22 msec. duration was applied to one of the pyramids and the responses recorded from *tibialis anticus* and *gastrocnemius*. Record *a* illustrates the response in the flexor muscle to a medium strong negative stimulus. *b* shows the facilitation of the response under catelectrotonic influence and *c* the depression under anelectrotonus. When the strength of the anelectrotonus is increased a small response can be observed in the extensor muscle (*d*) and with further increase a still greater response is obtained (*e*). Thus it is seen that parallel to the depression of the flexor response the anelectrotonus causes an increase of the excitability of the extensor system. The latencies in the records *d* and *e* show that the extensor discharges are break responses. Since at break the falling phase of the negative current pulse represents a potential change towards positivity, the extensor system is activated by a current direction normally most effective for this system.

A corresponding series of records with positive test shocks is shown in *f—k*. Record *f* shows the extensor response to a medium strong current and *g* the facilitation of this response by anodal polarisation. In *h* the strength of the catelectrotonus is such that the response is nearly abolished. With successive increase of the catelectrotonus, the depression of the extensor response becomes complete, at the same time as the excitability of the antagonistic system is increased so that first small and then large flexor discharges are obtained (records *i* and *k*). The results are easily repeatable and identical effects can be elicited also when the polarisation is applied through separate electrodes placed on the surface of the medulla.

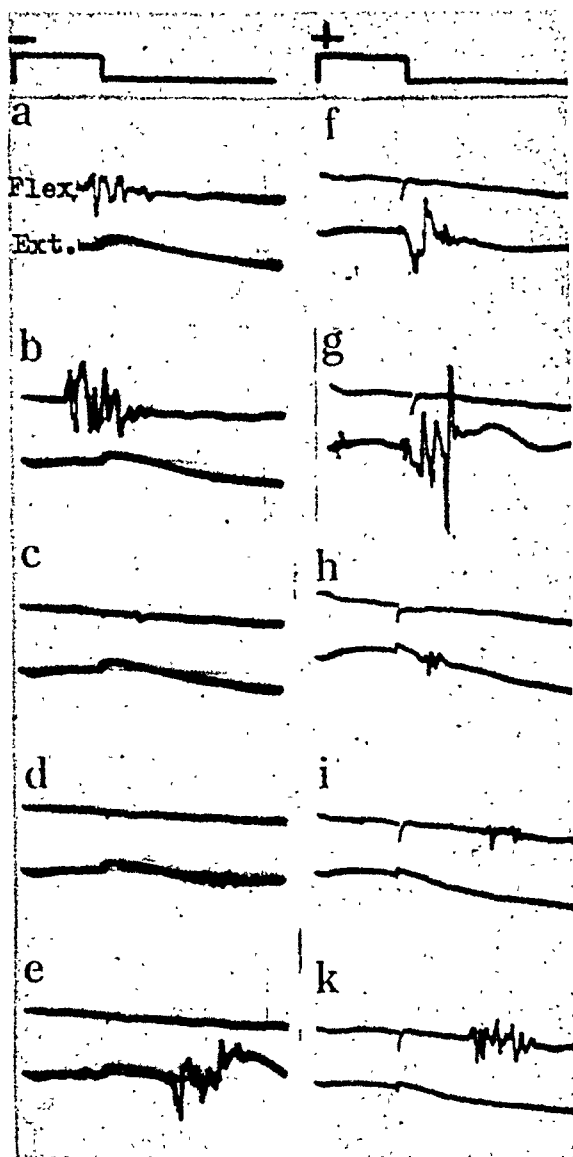


Fig. 7. Influence of constant polarisation on responses in *tibialis anticus* (Flex.) and *gastrocnemius* (Ext.) evoked by square wave stimulation of the medulla. Stimulus form marked at top of the series.

a—e negative test stimuli, f—k positive test stimuli.

a effect of test stimulus alone. b with negative polarisation through the inserted stimulating electrode.

c—e with positive polarisation of successively increasing strength.

f. positive test stimulus alone. g with positive polarisation.

h—k with negative polarisation of successively increasing strength. Further description in text.

This experiment is presented principally in order to show that central neurons are affected by electrotonus in the same way as the peripheral nerves. In a nerve under influence of catelectrotonus, the responses to negative test shocks are facilitated and those to positive shocks inhibited, while the effects under influence of anelectrotonus are diametrically opposite (see *e.g.* SKOGLUND 1945).

The experiment is also instructive because it shows that the state of polarisation of the central neurons plays a significant part in the excitation processes evoked by different stimuli.

Characteristics of the motor unit discharges evoked by direct current stimulation of the medulla.

In one experiment a pair of recording electrodes was placed in the pyramid caudally to the stimulating electrode so that the response of the neurons in the pyramidal tract could be directly observed. It was found that the central neurons responded to direct current stimulation with repetitive discharges having a rhythm very similar to the natural discharges described by ADRIAN and MORUZZI (1939). That direct currents correspond to physiological stimuli is also demonstrated by the fact that the activation of the motor units is the same as in voluntary contractions. Fig. 8 *a* shows the responses in a flexor muscle to a medullary stimulus which was increased very slowly in strength. The large motor unit begins with a frequency of about 5 per sec. and attains a frequency of 25—30 per sec. The small unit has a higher initial frequency but attains the same final frequency. Records *b* and *c* are continuous and show the typical discharges in *quadriceps* to a steadily increasing stimulus strength. The initial frequency of the lowest threshold unit is about 10 per sec.; with increased stimulus strength new units are activated simultaneously with frequency increase. The maximum frequency of the large spikes recorded in the beginning of record *c* is about 50 per sec. The different units drop out successively with decrease in stimulus strength and the first unit activated is the last to dis-

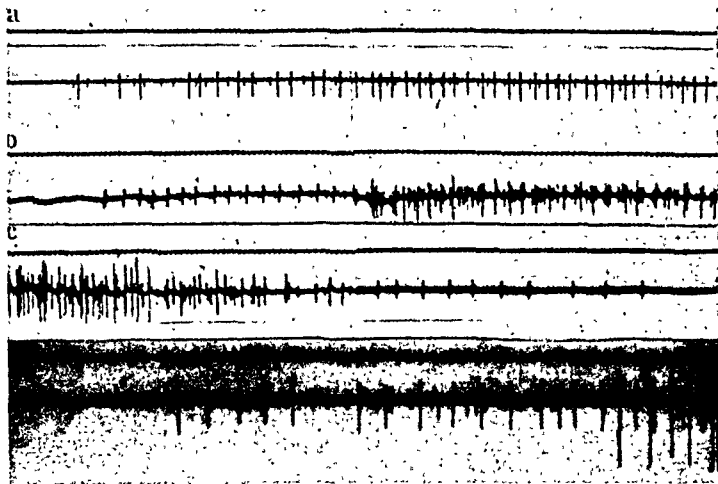


Fig. 8. Motor unit discharges to direct current stimulation of the medulla. Full description in text.

appear. Record *d*, from a flexor muscle, shows clearly that the artificially evoked contraction begins with a unit of small amplitude followed by units of progressively greater amplitudes. These results are in full agreement with the well-established findings in voluntary contractions (see KUGELBERG and SKOGLUND 1946).

These experiments gave the general impression that the discharges in the extensor muscles were of longer duration than in the flexor muscles when currents of the same relative strength were applied to the medulla. Therefore quantitative determinations of the duration of the flexor and extensor discharges were made. In one experiment the discharge of the lowest threshold element lasted about 1 sec. in the flexor and 5 sec. in the extensor muscle, when currents of 1.5 times the rheobasic strength were applied. In another experiment the duration of the responses to a current of twice the rheobase was 0.8 sec. in the flexor and 1.9 sec. in the extensor; the corresponding values for a current of 3 times the rheobase were 1.2 and 8.0 sec. respectively. A third experiment also showed significant differences between the adaptation times of the two systems. Of course no conclusions can be drawn whether this result is due to differences in excitability of the descending fibres or to different transmission processes in the spinal cord.

Discussion

The medulla oblongata was selected for the investigations intended to determine whether other parts of the motor system showed the same specific reactions as the spinal cord to stimulation with currents of opposite direction, because here the pyramidal tracts are near the surface and easily reached for stimulation.

It was in fact very easy to evoke these reciprocal phenomena by stimulation at the selected level of the motor system. However their analysis was complicated by various factors. Current spread is quite large and variable when surface electrodes are used and even with needle electrodes it is difficult to limit the stimulus effects only to the pyramidal tract when stronger stimuli are used. Other descending tracts are involved and different cell groups in the medulla such as the reticular formation may also be excited (cf. LLOYD 1941 a and b).

The investigations on cats regarding the association between the pyramidal and extrapyramidal systems and flexor and extensor functions are relatively few and the data incomplete (cf. TOWER 1935, MARSHALL 1936) which makes the analysis difficult.

The different explanations for the reciprocal phenomena in the spinal cord (see introduction) may also be applicable to experiments with medullary stimulation. A different orientation of the elements in relation to the current flow is possible if the flexor and extensor systems are to be found in different tracts or in different medullary cell groups. Some results have also been obtained which show that the locus of excitation is important for the dominance of the flexor and extensor responses. Thus it was observed in several experiments that flexor movements are more easily obtained from the cranial and medial parts of the medulla and extensor movements from the caudal and lateral.

In certain experiments the site of excitation has been definitely located in the pyramidal tract and it is difficult to believe that the different neurons are grouped within one and the same tract in such a way that they would be influenced differently by a

current of a given direction. It is more probable that the central neurons belonging to the flexor and extensor systems possess different excitability properties (cf. the differences in the characteristics of the peripheral motoneurons demonstrated by BERNHARD and THERMAN 1947). Especially the fact that, in the majority of the experiments, negative stimulus always elicits flexion while positive stimulus elicits extension seems to indicate this alternative. The opposite influence of constant polarisation on the excitable mechanisms of the systems may also be most easily explained by assuming different inherent properties of the two systems. Finally, the result that peripheral fibres with different properties can be selectively excited by currents of opposite direction (v. EULER and SKOGLUND 1947) shows that this assumption is not only speculative, but can be experimentally supported.

Summary

The descending motor tracts have been activated by stimulation at various points of the medulla oblongata with direct currents of opposite directions and the responses recorded in different muscles and nerves.

With the electrodes in given positions on the ventral surface of the medulla, stimulation in one direction causes principally flexor responses, whereas extensor responses predominate after reversal of the current. With a needle electrode inserted in the medulla and an indifferent electrode in the tissue, flexor movements are usually evoked when the active electrode is negative and extensor movements when it is positive. The thresholds for the two effects are about the same. Reciprocal inhibition can also be produced by artificial stimulation of the medulla.

Special experiments have been performed in order to determine where the excitation takes place. Low threshold areas from which typical reciprocal effects can be evoked are found at various points corresponding to both pyramidal and extrapyramidal tracts. Experiments with transection of the medulla also indicate that both these systems are involved.

The latencies of the peripheral discharges in the lumbar roots are the same when flexor and extensor systems are activated.

The excitability of the two systems has been studied under the influence of constant polarisation.

The motor unit activation caused by direct current stimulation of the medulla has been analysed and its similarity to natural discharges emphasized.

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Alternating Facilitation and Inhibition of the Extensor Muscle Activity in Decerebrate Cats

By C. G. BERNHARD *and* P. O. THERMAN*

Introduction

In 1902 FRÖHLICH and SHERRINGTON studied the inhibition of decerebrate rigidity using local stimulation of the spinal cord. On decerebrate cats they made a transection of the spinal cord in the upper lumbar or lower thoracic region. Iterative stimulation, with a small pointed electrode placed on the surface central to the transection within the ventrolateral white column, evoked relaxation of the extensors of the ipsilateral foreleg. The same effect could be evoked by stimulation of different nerves of the hind leg.

We started the experiments in order to confirm the observations of FRÖHLICH and SHERRINGTON and we also studied the electrical activity in the triceps muscle of the foreleg using local stimulation of the surface of the transected spinal cord. It was observed that stimulation of an area close to the border of the anterior and lateral grey columns, obviously corresponding to the propriospinal tracts, as well as stimulation of the sciatic nerves evokes a periodic alternation between activity and inactivity in the triceps muscle with a certain time relation to the stimulus. This rhythmical activity indicates periodical excitability changes in the spinal cord of entirely different characteristics than those previously described by BERNHARD (1945).

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Method

Experiments were carried out on decerebrate cats. In one series the spinal cord was transected in the lower thoracic region and three pair of roots above the section were cut. The cut face of the upper part of the transected cord was stimulated with square shocks via a needle electrode (cf. FRÖHLICH and SHERRINGTON 1902). In another series, the spinal cord was left intact and the stimuli were applied to the sciatic nerve. Records of the action potentials from the triceps muscle of the foreleg were obtained with concentric needle electrodes. The muscle discharge was recorded from two needles inserted at different points in the muscle (two records on each picture). In most experiments the foreleg was fixed and the distal part of the triceps muscle was freed so that the tendon could be fixed to a myograph stand. Condensor-coupled amplifiers and a double-ray cathode oscillograph were used.

Results

FRÖHLICH's and SHERRINGTON's phenomenon was confirmed in preliminary experiments, in which the muscles of the foreleg were left intact. The interruption of the extensor rigidity in the foreleg could easily be observed when a small limited area in the ventrolateral white column on the ipsilateral side of the spinal cord was stimulated. However, the exact position of the stimulating electrode is of great importance in eliciting a rapid effect of relaxation. By placing the electrode in the most favourable position within the area described by FRÖHLICH and SHERRINGTON relaxation of the ipsilateral foreleg was obvious after only one or a few stimuli even with a low frequency.

We found, further, that stimulation, with frequencies less than 10 per sec. of an area quite near and lateral to the anterior and lateral columns, often evoked an effect which might be described as a pulsation between contraction and relaxation of the ipsilateral extensor muscles. In such cases an increase of the stimulus fre-

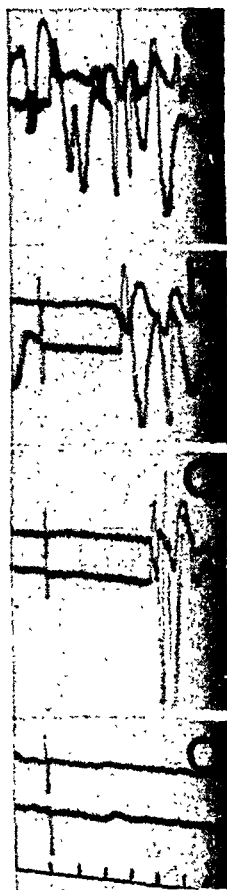


Fig. 1. The effect of iterative stimulation (6 per sec.) of an area within the left ventrolateral white column (upper lumbar segment) on the decerebrate activity of the triceps muscle of the left foreleg. (Action potentials, in all figures, from two different points in the muscle simultaneously). *a* extensor activity before stimulation, *b* after the first, *c* after the second and *d* after the third stimulus. Time in 10 msec.

quency often tended to evoke a dominating relaxation after varying periods of stimulation.

FRÖHLICH's and SHERRINGTON's phenomenon of inhibition is clearly demonstrated by recording the electrical activity of the triceps muscle (Fig. 1). Record *a* shows the continuous muscle activity at constant tension and *b—d* the effect of the first, second and third stimuli (frequency 6 per sec.). The stimuli are synchronised to the sweep and the stimulus artefact is visible to the left on each record.

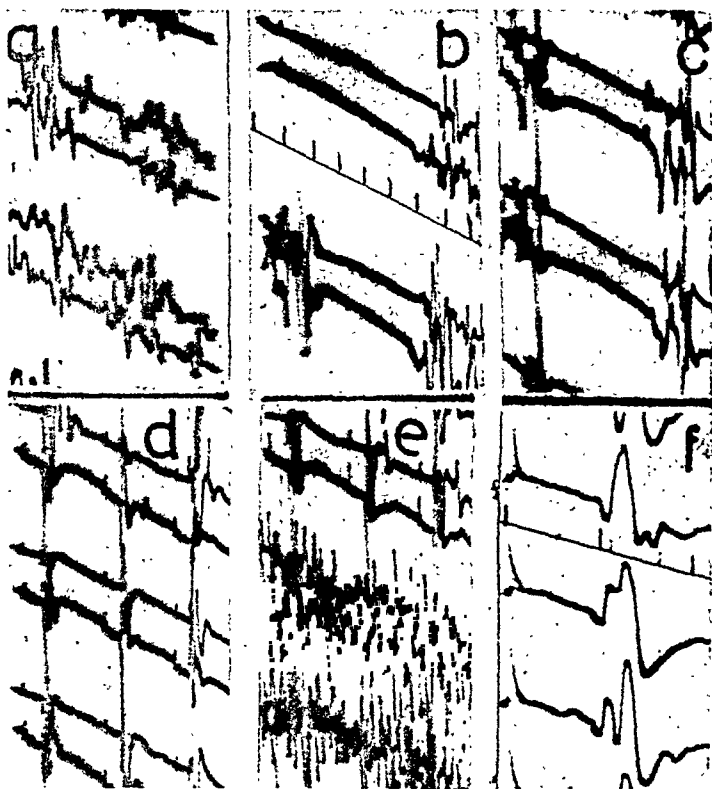


Fig. 2. The effect of iterative stimulation of the spinal cord near the border of the intermediate grey matter (left side, upper lumbar segment) on the triceps muscle activity of the left foreleg. *a* before stimulation, *b* after first and second stimulus (frequency 12 per sec.), *c* after the 12th—14th stimulus (same frequency), *d* frequency 40 per sec., *e* same as in *d* and after cessation of stimulation (lower records), *f* same as in *d* but with higher sweep velocity. Time in 10 msec.

After the first stimulus (*b*) there is a «silent period» of about 30 msec. After the second stimulus the period of inactivity is of still longer duration (about 40 msec.) and the succeeding discharge well synchronised. However, after the third stimulus (*d*) the activity has entirely disappeared.

As mentioned above, a slight displacement of the stimulating electrode towards the border of the grey matter changes the effect, as is shown in Fig. 2, where no permanent inhibition of the triceps activity could be obtained. Fig. 2 *a* illustrates the activity prior to the beginning of the iterative stimuli (12 per sec.). The upper records in Fig. 2 *b* illustrate the effect of the first stimulus. The

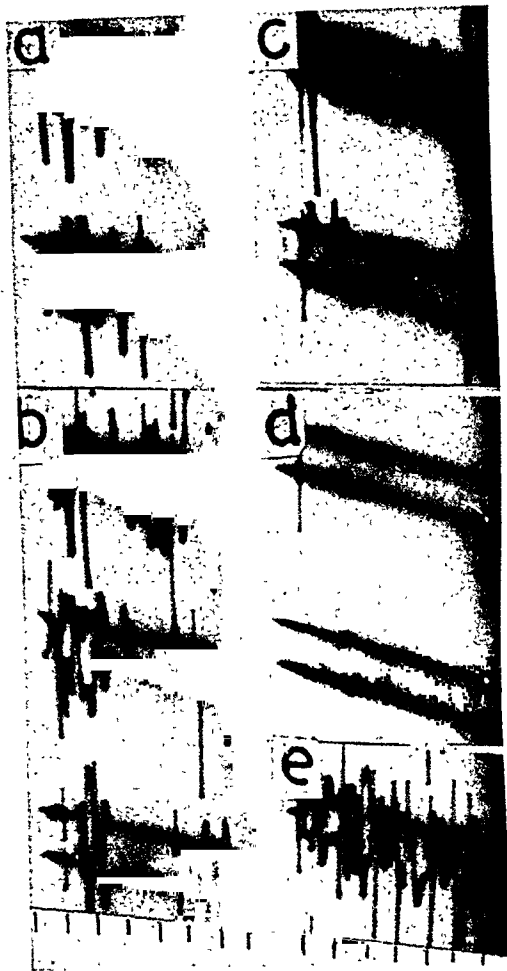


Fig. 3. The effect of iterative stimulation (frequency 3 per sec.) of the right sciatic nerve on the activity of the left triceps muscle. *a* before stimulation (also upper records in *b*), *b* (lower records) after first and second stimulus, *c* after 7th and 8th and *d* after 10th and 11th stimulus, *e* activity after cessation of stimulation. Time in 10 msec.

following inactivity has a duration of about 60 msec. The records in Fig. 2 *c* clearly demonstrate that the rhythmic activity following each stimulus is characterised by two maxima of activity (after about 10 and 50 msec. respectively) separated by a period of inactivity. Records in Fig. 2 *d* and *e* show the effect with higher frequency. The frequency is about 40 per sec. and three stimuli occur on each sweep. It can be seen that the first period of activity after each stimulus is greatly reduced when it falls within

the period of inactivity evoked by the preceding stimulus. Thus each period of activity is represented by only one muscle spike. After cessation of the stimulation the non-periodic activity returns but now with greater intensity than before (rebound, cf. SHERRINGTON 1907). Records in Fig. 2 *f* are obtained with higher sweep velocity and show that the latency of the initial muscle discharge is about 10 msec.

The same type of rhythmic activity may be obtained from the triceps muscle of the foreleg when either one of the sciatic nerves is stimulated. Record 3 *a* shows the typical continuous extensor muscle activity in the decerebrate state with constant tension of the muscle. Records 3 *b* and *c* show the potential pictures at the 1st, 2nd, 7th and 8th stimuli (3 stimuli per sec. applied to the contralateral sciatic nerve), and clearly demonstrate the rhythmic activity following each stimulus. For instance, in the lowest record of Fig. 3 *b*, the stimulus is followed by an activity after about 8 msec., subsequently followed by a period of inactivity. This is then succeeded by a new period of activity occurring about 40 msec. after stimulation (cf. Fig. 2 *c*). The duration of the period of inactivity increases successively after the following stimuli (Fig. 3 *c* and upper records in 3 *d*) and after 11 stimuli the activity has completely disappeared (lower records in Fig. 3 *d*). Record 3 *e* obtained after cessation of stimulation again shows the rebound (cf. Fig. 2 *e*).

The fact that a discharge, produced during a period of inactivity evoked by the preceding stimulus, is reduced (Fig. 2 *d*) indicates that there is actually an inhibition of the extensor activity. This is also confirmed by the fact that the intense background activity prior to the iterative stimulation (2 *a* and 3 *a*), completely drops out during the period of inactivity. The experiment which is illustrated in Fig. 4 indicates that the inhibition of the triceps activity is maximal during the middle part of the period of inactivity. In this case stimulation of the contralateral sciatic nerve produces typical rhythmic activity (4 *a*). If the tension of the triceps muscle itself or, of a synergist, is increased, the proprioceptive impulse in-flow will reflexively produce an increase of the muscle discharge. Records 4 *b* and *c* show that the activity increases in such a way, that the periods of activity are

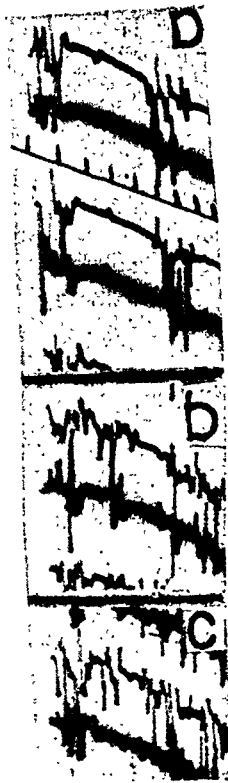


Fig. 4. Same as in Fig. 3 c combined with increasing tension of the triceps muscle (b and c). Time in 10 msec.

spread out, *i. e.* the gap between them is cut off from both sides. This means that the inhibition is most pronounced in the middle part of the silent period where the resistance to myotatically produced excitation is strongest.

Discussion

Stimulation, within the area lateral to the anterior and intermediate grey matter, obviously engages propriospinal fibres mediating long spinal reflexes (*Fasciculus proprius*, see *e. g.* RANSON and CLARK 1947). When this area or the sciatic nerve is stimulated the continuous muscle discharge changes to a rhythmic activity, the different phases of which have a definite time relationship to the stimulus. Two phases of facilitation are

separated by a period of inhibition. The stimulus may evoke a multisynaptically transmitted discharge (record 2 f) which, then, may represent the first period of activity. As pointed out by LLOYD, the long spinal reflex discharge has great variability as is also shown in our experiments (no initial discharge in Fig. 2 b, but well developed after iterative stimulation especially in Fig. 2 f). The latency tends to be about 10 msec. (cf LLOYD 1942). Thus, the experiments indicate that stimulation of the propriospinal tracts or the sciatic nerves elicits a periodic excitation of the extensor motoneurons. Diagram 5 illustrates schematically these cyclic variations of the central excitation of the extensor motoneurons. The crests of the curve represent facilitation of the extensor neurons and the dip corresponds roughly to the instant when the inhibition of the myotatically evoked discharges is most pronounced.

The question is, whether the later phases of the cyclic changes of excitation are dependent on intraspinal processes or if they are evoked through influence from the periphery, *i.e.* proprioceptive impulses from the antagonists and synergists of the foreleg which take part in the activity. In an attempt to clarify this problem, preliminary experiments were performed, in which denervation and anaesthetisation of different muscles were used. The experiments suggested that the cyclic changes of excitation depend on intraspinal processes, but that they may be influenced from the proprioceptive in-flow from contracting muscles. The problem has later been further analysed (see BERNHARD and THERMAN 1947). In experiments on descending long spinal reflex activity LLOYD (1942) studied the influence of brachial plexus volleys on the excitability of lumbar two-neuron arcs and demonstrated an early inhibition lasting for about 10 msec. followed by a facilitation of about 35 msec. Our results, however, can not directly be compared with those of LLOYD because the experiments, described above show the influence of ascending tract volleys on the extensor system only.

While stimulation of the sciatic nerves as well as the propriospinal tracts usually is followed by alternating facilitation and inhibition of the extensor activity of the foreleg, stimulation of the area described by FRÖHLICH and SHERRINGTON produces a

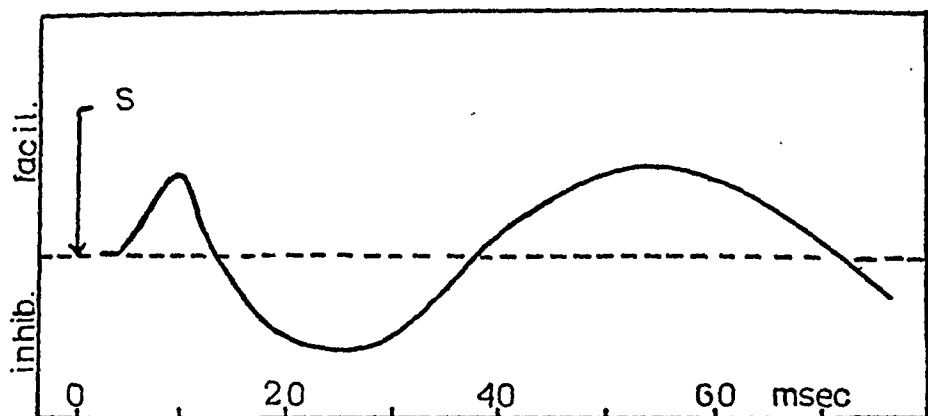


Fig. 5. The curve illustrates the periodical excitation of the extensor motor neurones of the foreleg following a single stimulus (S) applied to the ipsilateral sciatic nerve or the propriospinal tracts. Upward deflection indicates facilitation and downward deflection inhibition. See text.

complete and long-lasting inhibition of the extensor activity. Fig. 1 illustrates how this inhibition develops. The synchronised discharge, which is clearly visible in Fig. 1 c, seems to correspond to the second maximum of activity, following stimulation of the propriospinal tracts (Fig. 2) or the sciatic nerves (Fig. 3 and 4). However, initial activity never occurs, and complete inhibition develops after a few or only one stimulus. These facts indicate that the stimulating needle touches pathways mediating impulses with selective inhibitory action on the extensor reflex system of the ipsilateral foreleg as pointed out by FRÖHLICH and SHERRINGTON. One or a few stimuli, applied to a limited area within the ventrolateral white column obviously, evoke long-lasting spinal processes accompanied by prolonged inhibition of the extensor activity.

Summary

1. Stimulation of the ascending propriospinal tracts or the sciatic nerve evokes a periodic alternation between facilitation and inhibition of the decerebrate extensor activity of the ipsilateral foreleg, the different phases having a certain time relationship

to the stimulus. The excitability changes seem to depend on long-lasting intraspinal processes.

2. Stimulation of the fairly limited area within the ventrolateral white column (described by FRÖHLICH and SHERRINGTON) evokes inhibition of the decerebrate extensor activity, which has been studied myographically. It has been shown how the application of a few or even one stimulus may evoke a profound inhibition of the ipsilateral extensor activity. The effect evoked from these fibres thus has entirely different characteristics from that described above. Impulse volleys in this tract seem to evoke even more long-lasting intraspinal processes accompanied by inhibition of extensor activity.

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Rhythmical Activity of Motor Units in Myotatic Reflexes

By C. G. BERNHARD *and* P. O. THERMAN*

Introduction

In a previous paper we demonstrated how the continuous extensor activity of the forelegs in the decerebrate state changes to grouped activity after stimulation of the sciatic nerve, the different groups of discharge having a certain time relationship to the stimulus (BERNHARD and THERMAN 1947). We also observed that passive movements of the foreleg evoke grouped activity in the triceps muscle. The experiments to be described below represent a further analysis of this observation and they show how the muscle discharge in the decerebrate state is transformed into a synchronised rhythmic activity during changes in the local proprioceptive in-flow. Further experiments were performed on different muscles in order to show how the rhythmical activity from different units participates in the building up of this synchronised discharge.

Methods

The cats were decerebrated and fastened with clamps to a stand in a heated, shielded box. In those experiments, which were performed in order to study the changes of the persistent extensor activity, cats were used which exhibited a steady and even extensor tone (transection at intercollicular level). The purpose

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of these experiments also justifies the choice of the foreleg extensors as the main object for study. The head was fixed in a slightly raised position. Care was taken not to change the exteroceptive and extrasegmental proprioceptive impulse in-flow. In other experiments the persistent flexor activity was analysed. The transection was then made in front of the anterior colliculi in order to obtain a persisting posture of the flexors (see *e. g.* DENNY-BROWN, ECCLES and LIDDELL, 1929). In some of the experiments the muscles of the foreleg were intact, but in most of them the biceps and triceps were distally prepared free. The leg was fixed with drills and the tendons were fastened to a myograph stand, so that the muscles could be independently stretched. The same method was used in experiments on muscles of the hind leg. Recording of the action potentials from the muscles was made with concentric needle electrodes or double, enamelled steel needles, the points of which were free. Two condenser-coupled amplifiers were used, connected to a double-ray cathode oscillograph.

Results

The mass activity, in the decerebrate state, from the intact triceps muscle is shown in Fig. 1 *a* (cf. also 2 *a*, *e*, *f*, *h*, and *k*). By flexing or extending the foreleg at the elbow, the proprioceptive in-flow from the triceps muscle is increased respectively decreased evoking a reflexively conditioned increase or decrease of the discharge from the muscle. However, during the change of the proprioceptive in-flow the muscle activity always passes through a phase characterised by pronounced grouped activity (Fig. 1 *b*). When the new position has been taken the continuous activity returns (1 *c*). When, as in this case, all the muscles acting at the elbow joint are intact, the change in central excitability is dependent on the change in the proprioceptive in-flow not only from the triceps muscle itself, but also on changes from the other muscles which are involved in the movement.

The following experiments were performed in order to determine the part played by the triceps muscle itself, a synergist or an antagonist in the elicitation of the grouped activity.

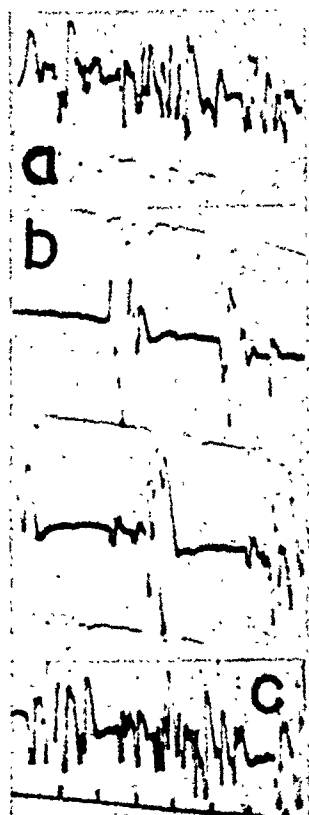


Fig. 1. Action potentials in decerebrate rigidity from the triceps muscle of the foreleg (intact muscles). *a* at full extension, *b* during slight passive flexion, *c* after flexion. Time in 10 msec.

With moderate and constant tension in the distally freed triceps muscle a continuous complex spike activity is recorded (Fig. 2 *a*, *e*, *f*, *h* and *k*). In *f*, *h* and *k* the activity has been simultaneously led off from two different points in the muscle (represented by the two sweep pictures close to each other). As may be expected there are marked differences between the two recording points with respect to the distribution of the muscle spikes.

Records 2 *b* and *c* show the change in the impulse discharge during decreasing tension of the triceps muscle. Thus, when the proprioceptive impulse in-flow from the muscle itself is reduced the activity successively decreases and drops out (lower tracings in Fig. 2 *c*). It is typical, however, that during the decrease in tension the disappearance of impulses is not evenly spaced but is

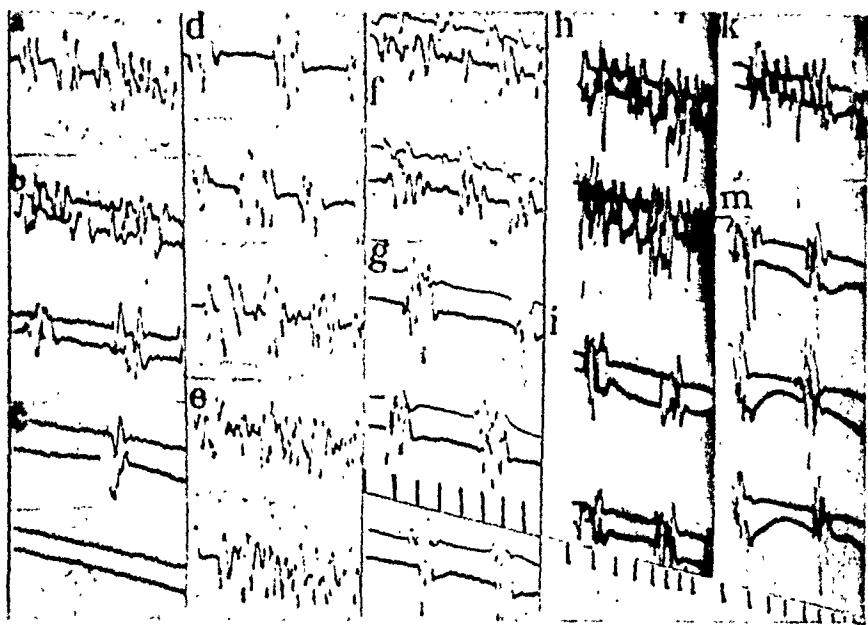


Fig. 2. Action potentials from the triceps muscle during *a—c* decreasing tension, *d—e* increasing tension; *f—g* increasing tension in the neighbouring extensor, *h—i* increasing tension in biceps, *k—m* pinching of biceps. Time in 10 msec. (The time scale on *g* refers also to records *a—f*).

characterised by grouped activity (beginning in 2 *b* upper tracings, and very obvious in 1 *b* lower tracings).

If, instead, the triceps muscle is stretched from complete relaxation (no activity as in Fig. 2 *c*, lower tracings) the activity again passes a phase characterised by marked grouped activity (*d—e*).

Changes of the tension in an extensor to the same joint also produce grouped activity in the triceps muscle. In records 2 *f—g* the tension in the triceps muscle has been kept constant. Record 2 *f* is obtained at moderate tension in the neighbouring extensor. When the tension in the other extensor is increased, grouped activity again occurs in the triceps muscle (2 *g*).

We then studied the influence, on the triceps muscle activity, of changes in the tension of an antagonist (the biceps muscle; records *h—i*). The continuous impulse discharge in the triceps muscle (2 *h*) is replaced by grouped action when the antagonist is stretched (2 *i*). The same typical picture of grouped activity

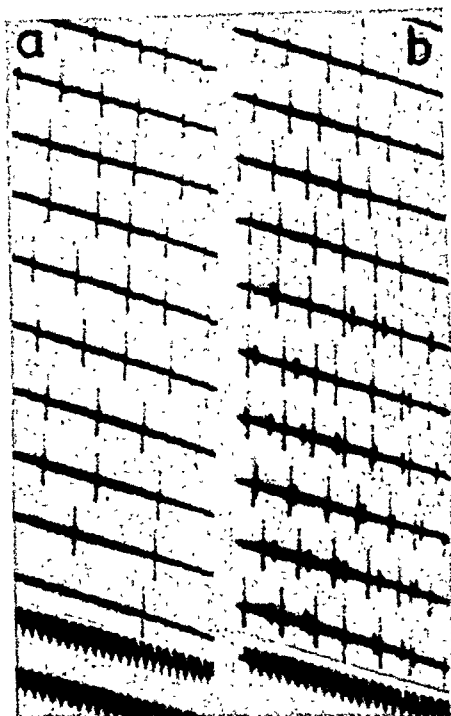


Fig. 3. Rhythmically discharging units from the triceps muscle during; *a* decreasing tension, *b* increasing tension. Time in 10 msec.

is obtained by pinching the biceps muscle (*k—m*). In these two latter experiments indirect influence from the extensor muscles was eliminated by freeing them from their distal points of attachment.

Attempts have also been made to evoke grouped activity in the triceps muscle by changing the suprasegmental proprioceptive in-flow. However, moving the cat's head up and down in order to change the proprioceptive in-flow from the neck muscles does not evoke any obvious grouped action provided that secondary influences from the foreleg muscles are prevented.

The frequency of the rhythmical triceps discharge, evoked in these various ways, tends to be about 30 per sec. *i. e.* the interval between two activity maxima is approximately 30 msec.

The fact, that the complex muscle spikes, in the case of grouped activity, have a much higher amplitude (see records 1 *b*, 2 *b*, *g* and *m*) indicates that the grouped activity actually depends on a synchronisation of activity from different units. This is further

supported by the fact that the potential groups recorded from different points in the muscle occur simultaneously despite the fact that in the state of continuous activity the distribution of impulses from the two recording points is different (see 2 *b*, *g*, *i* and *m*).

In order to obtain further information concerning synchronisation, evoked by changes in the proprioceptive impulse in-flow, we studied the activity of single units, during very slight variations in the tension of the muscles. Care was taken to insert the needle electrodes in such a way that spikes of equal amplitude were obtained with a slight tension in the muscle. (Fig. 3 and 8.) We thus studied units with low threshold to myotatic stimulation. Fig. 3 shows the well known changes of frequency in rhythmically discharging units by changing the myotatic influence through reduction and increase of the muscle tension. The experiments are presented to illustrate the observations: 1) that the »low threshold unit» can not maintain a rhythmical discharge with a low frequency but drops out (3 *a*), 2) that the increase of frequency of the same unit is limited also at increased tension (3 *b*). At higher tension a new unit is brought into play with approximately the same frequency (Fig. 3 *B*). At still higher tension more units are involved, but the picture then becomes so complicated (cf. Figs. 1 and 2) that the rhythms of the individual units can not be analysed.

Diagram 4 shows how the intervals between the spikes of a rhythmically discharging unit with low threshold to myotatic stimulation (open circles, curve I) are lengthened by reducing the muscle tension (at A). It is true that at lower tension the intervals are lengthened but a low frequency can not be maintained and the spike entirely drops out. With increasing tension (at B) the rhythmic discharge begins again and the spike interval approaches the initial value. At C the muscle tension is further increased thus reducing the spike interval to a slightly lower value and a second spike is brought into play (filled circles, curve II). When the muscle is again relaxed the second spike drops out, followed by the disappearance of the first spike. Thus, under the given conditions, the unit with low threshold to myotatic stimulation tends to work within a fairly limited »frequency band».

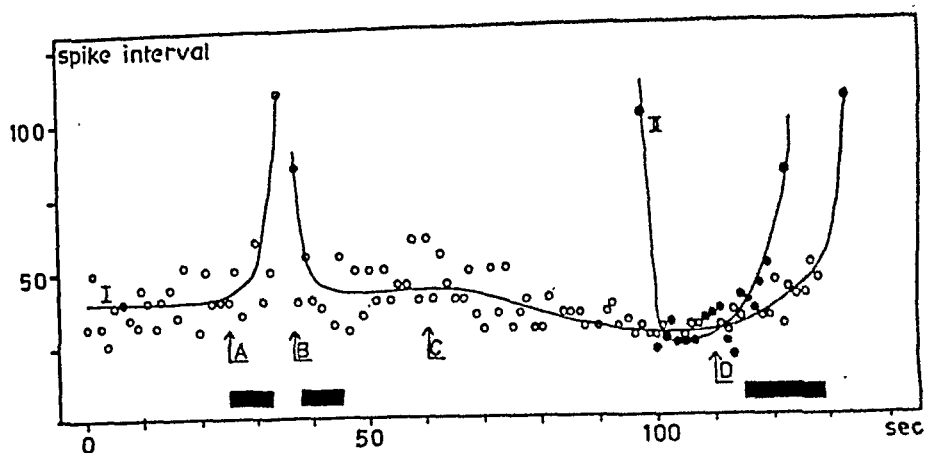


Fig. 4. Intervals (in msec. on the ordinate) between the spikes of a rhythmically discharging unit in the triceps muscle during varying tension. Time of the experimental procedure on the abscissa. Open circles indicate intervals between spikes of a unit with low threshold to stretch; filled circles of a unit with somewhat higher threshold. Arrows at A and D indicate decrease in tension; at B and C increase of tension. Filled columns at the bottom of the diagram indicate the periods during which double responses occur.

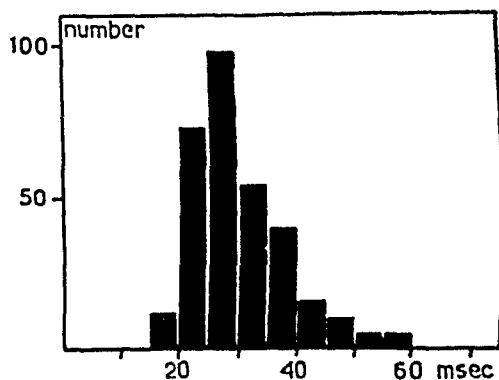


Fig. 5. Distribution of intervals between spikes of a rhythmically discharging unit of the biceps muscle during myotatic influence. Each class covers 10 msec. (on the abscissa). Number of measured intervals in the different classes on the ordinate.

We have determined such frequency bands in different muscles for units with low threshold to myotatic stimulation. Fig. 5 shows the material (256 values) from one experiment on the triceps muscle of the foreleg. The columns represent the number of measured spike intervals (on the ordinate) in classes of equal size, each one including 5 msec. (along the abscissa). The maximum lies between the interval values 25 and 30 msec. In order

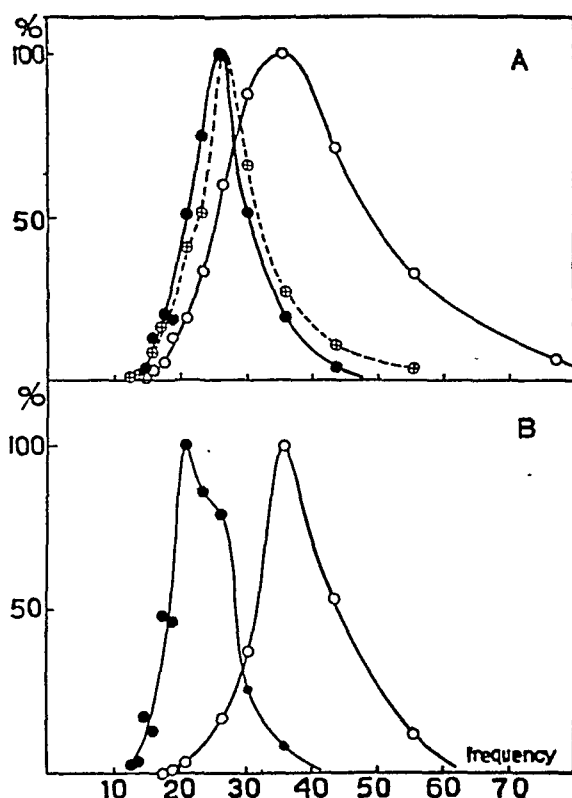


Fig. 6. Frequency bands from rhythmically discharging low threshold units of different muscles, A hind leg, filled circles quadriceps, open circles adductor longus, circles with cross gastrocnemius; B foreleg, open circles biceps, filled circles triceps. For further description see text.

to express the frequency band the inverted values of the spike intervals are plotted along the abscissa (Fig. 6). The number of values in each class have been plotted along the ordinate in per cent of the maximum column. Fig. 6 A shows the frequency bands for the gastrocnemius (crossed circles), quadriceps (solid circles) and adductor longus (open circles) of the hind leg, while 6 B illustrates the frequency bands for the triceps (solid circles) and biceps (open circles) of the foreleg (300—1,000 values for each curve).

Having studied the behaviour of the individual unit, we were able to analyse the development of synchronisation of different units which is illustrated in Fig. 3 b. When the small muscle spike

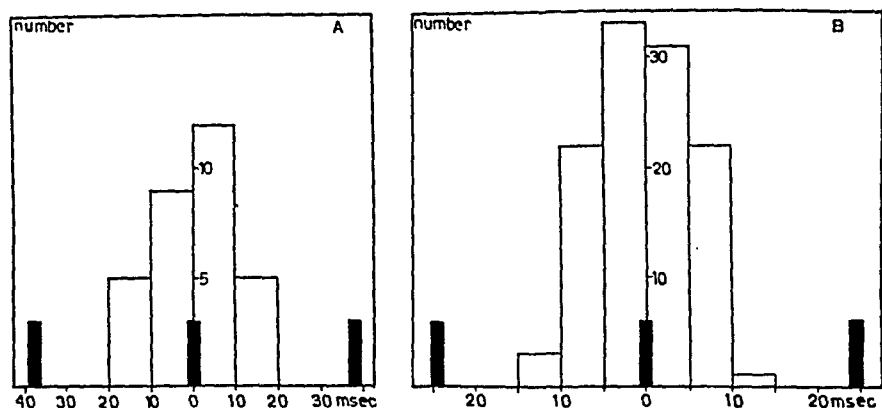


Fig. 7. Diagram showing the building up of a synchronised rhythmical activity by two different muscle units. For further description see text.

is brought into activity, it tends to appear in the neighbourhood of the large spike. If the average for the intervals between the large spikes is calculated from the last 10 records the value 38.6 msec. is obtained. In addition the distance has been measured between each small spike and the *nearest* large spike. The observations are graphically illustrated in Fig. 7 A. The distances between the three thin solid columns represent the average for the intervals between the large muscle spikes. The broad columns to the left of zero on the abscissa indicate the number of small spikes which appear during the periods 0—10 msec. respectively 11—20 msec. before the nearest large muscle spike; while the columns to the right indicate the number of small spikes occurring during the periods 0—10 msec. respectively 11—20 msec. after the nearest large muscle spike.

Diagram 7 B, based on a larger material obtained under similar conditions from the biceps muscle (112 values; each class including 5 msec.), confirms the observation that when units with high threshold to myotatic stimulation are brought into play their spike discharges tend to keep close to those of a unit already discharging.

In most of our experiments we also found the »double response» of the same unit, which was first described by ECCLES and HOFF (1932). This phenomenon usually occurs when the tension of the muscle passes the threshold value for rhythmic activity (Fig. 8).

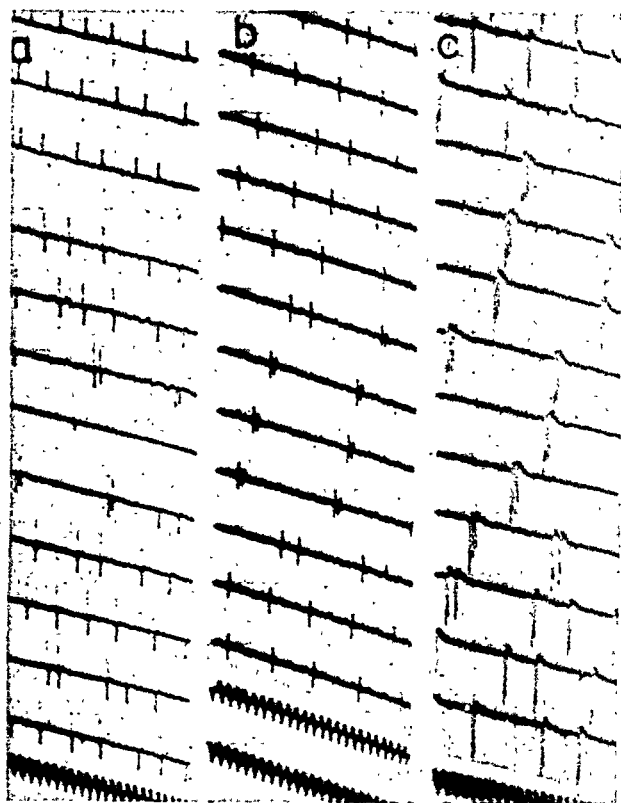


Fig. 8. Doubling and tripling in, *a* triceps (foreleg), *b* tibialis anterior, *c* gastrocnemius during temporary decrease of muscle tension. Time in 10 msec.

In 8 *c* both »doubling» and »tripling» occurs. The doubling and tripling is characterised by an initial spike of normal size followed by a second smaller and sometimes a third still smaller spike. It is not necessary to comment further the difference between the picture of repetitive discharges from the same unit and that of two simultaneously discharging units (Fig. 3 *b*). In Fig. 4 the horizontal columns at the bottom of the diagram show the periods during which this phenomenon occurs.

Discussion

In the well known condition of decerebrate rigidity (SHERINGTON 1898) the extensor muscles involved produce a persistent contraction, the electrical activity of which was first shown

by DUSSER DE BARENNE (1911) and BUYTENDIJK (1912). The persistent regular, rhythmical activity of single units in the decerebrate state at passive tension of the muscle was first demonstrated by DENNY-BROWN (1929) and by ADRIAN and BRONK (1929). DENNY-BROWN states in his paper on postural reflexes in decerebrate cats that »each unit is sudden in onset of discharge and keeps an approximately even rate despite the continuously increasing tension». This observation is further supported by our experiments (Figs. 4, 5 and 6), which show that in the decerebrate state, a single unit under myotatic influence tends to work within a fairly limited frequency range.

An increase of the proprioceptive excitation (caused by an increase of the passive muscle tension) is followed by »recruitment» of new motor units (LIDDELL and SHERRINGTON 1923), each preserving an approximately even rate of discharge (cf. DENNY-BROWN 1929). It is obvious (see Figs. 3 *b* and 7) that the spikes of single units which are brought into play by increasing the muscle tension tend to keep close to the spikes of the units which — because of their lower threshold — are already discharging. Thus the experiments show how the synchronisation of the rhythmical activity is built up by two different units.

In decerebrate rigidity the mass activity of the extensors has a tendency to grouped action. The above experiments show that the synchronisation of the different rhythmically acting units is especially pronounced during the change in the proprioceptive in-flow from the muscle itself, the antagonists or the synergists (Figs. 1 and 2). The experiments on single units discussed above represent an analysis of this phenomenon. It may be pointed out that the frequency of the grouped mass activity in Fig. 2 is of the same order as that found in experiments on single units (Figs. 4, 6 *B* and 7 *A*). The rhythmical discharge of the motoneurons is dependent on the intensity of the persisting excitatory influence, and it is obvious that in the decerebrate state a change in that fraction of excitation, which depends on the local proprioceptive in-flow, favours the synchronisation of the rhythmical activity.

The synchronised activity is easy to separate from the multiple spike activity from the same unit (Fig. 8) *i. e.* the double response.

This phenomenon is known from earlier investigations on reflex activity (ECCLES and HOFF 1932; HOFF and GRANT 1944) and has been shown to occur also during quick voluntary movements (GILSON and MILLS 1941). Doubling and tripling occur by varying the proprioceptive in-flow near the threshold value of myotatic stimulation (cf. HOFF and GRANT 1941).

The difference between the response from the individual units during changes of the proprioceptive influence in the decerebrate state and the response during changes in the contraction under voluntary control must be emphasized. ADRIAN and BRONK (1929) were the first to study the response of single units during voluntary contraction; later many investigations have been made on the same subject (see *e. g.* SMITH 1934, LINDSLEY 1935, HOEFER and PUTNAM 1939, SEYFFARTH 1940, GILSON and MILLS 1941, WEDELL, FEINSTEIN and PATTLE 1945, KUGELBERG and SKOGLUND 1946). A change in the excitatory influence on the motoneurons from cortical levels — as in the case of changing voluntary contraction — is followed by an uniform change in the the frequency of the different units in contrast to the fixed frequencies during varying myotatic influence in the decerebrate state. In addition the tendency to synchronised activity is not so pronounced in the case of voluntary control. It is true that *e. g.* STETSON and BOUMAN (1935) have reported grouped activity (50 per sec.) from the muscles of the forearm during tapping movements of the hand. Most investigations, however, show that the various units during changes in voluntary contraction discharge asynchronously (see *e. g.* GILSON and MILLS 1945, KUGELBERG and SKOGLUND 1946). It has also been demonstrated that, during increasing voluntary excitation, units giving small amplitude potentials are activated before those giving larger spikes (SMITH 1934, DENNY-BROWN and PENNYBACKER 1938, KUGELBERG and SKOGLUND 1946). This is not the case during increasing myotatic stimulation in the decerebrate state (Fig. 3).

The phenomenon of synchronisation during myotatic influence in the decerebrate state has to be borne in mind when considering the electromyographic studies of those nerve diseases in which synchronised activity has been found (see *e. g.* HOEFER and PUTNAM, 1940, BUCHTHAL and CLEMMESSEN, 1943 and 1946).

The curves in Fig. 6, representing frequency bands of low threshold units in different muscles, may indicate the possible existence of differences in the characteristics of the motoneurons of different systems *e. g.* extensors and flexors. We have the general impression that extensor units discharge with lower frequencies than flexor units in the state of persistent extensor or flexor posture. Still lower frequencies in the persistent rhythmical discharge of extensors are demonstrated in the paper by DENNY-BROWN (soleus), ADRIAN and BRONK (quadriceps). Further investigations are, however, necessary in order to demonstrate whether there is actually a definite difference between the characteristics of antagonistic motoneurons or not.

Summary

1. The experiments show that in the decerebrate state the persistent extensor muscle discharge is transformed into a synchronised rhythmic activity during changes in the proprioceptive in-flow from 1) the muscle itself 2) a synergist 3) an antagonist.

2. In order to analyse this phenomenon experiments were performed in which the rhythmic activity of single motor units in different muscles was studied. It was shown that in the decerebrate state units with low threshold to myotatic stimulation tend to discharge within relatively limited frequency ranges during varying proprioceptive excitation (evoked by changing the muscle tension). Descriptions of frequency bands from different muscles are presented.

3. Further experiments demonstrate how the synchronisation of the rhythmical activity from two different units is built up during the increase of the muscle tension.

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Reciprocal Facilitation and Inhibition following a Single Afferent Volley

By C. G. BERNHARD *and* P. O. THERMAN*

Introduction

It has been demonstrated that stimulation of either one of the sciatic nerves is followed by alternating facilitation and inhibition of the activity in the triceps muscle of the foreleg, the different phases of excitation having a fixed time relationship to the stimulus (BERNHARD and THERMAN 1947 b).

The purpose of the present investigation is to gain further information on the slow rhythmical changes of the excitation in the extensor and flexor motoneurons following a single afferent volley. The experiments to be described show how periods of facilitation and inhibition alternate in the antagonistic motor systems of the forelegs following a stimulus applied to the sciatic nerve. Similar phenomena are demonstrated by stimulation of a nerve of the foreleg. Preliminary experiments show that the slow ventral root potentials may have a rhythmical course, the different phases of which may be correlated to the changes of excitation mentioned above.

Methods

Decerebrate cats were used having pronounced rigidity of the forelegs. The triceps and biceps muscles of the forelegs were freed distally and their tendons fixed to a myograph stand. The action

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potentials from the muscles were led off with needle electrodes and recorded with condenser-coupled amplifiers and double-ray cathode oscillographs. In some experiments the action potentials were simultaneously registered from the triceps and biceps muscles of both forelegs. Different nerves were stimulated with electrical shocks, which were synchronised to the sweep.

Results

1. Electrical mass activity in antagonistic muscles of the foreleg.

In cats which — because of the transection at supracollicular level — develop a persistent posture of both flexors and extensors in the forelegs, a continuous potential activity can be recorded simultaneously from the biceps and triceps when they are slightly stretched. When a single stimulus is applied to the contralateral or ipsilateral sciatic nerve the continuous activity in the two antagonists is transformed into grouped activity. It is typical that the flexor is active during the periods when the extensor is silent and vice versa. Fig. 1 illustrates the common type of response (ipsilateral sciatic nerve stimulated) provided that both muscles are under continuous myotatic excitation during the experiment.

An increase of the tension in the proprioceptive in-flow from the triceps evoked by an increase of the tension in the muscle is followed by an increase of activity in the same muscle and a reduction of the activity in the antagonist. It has previously been demonstrated that during the increase of proprioceptive excitation the periods of activity in the *same* muscle become prolonged so that the dividing gaps are shortened (BERNHARD and THERMAN 1947 b). It is easy to show that at the same time the opposite occurs in the antagonist, *i.e.* a prolongation of the silent periods at the expense of those of activity. With sufficient tension in the triceps the activity entirely disappears in the biceps muscle. The last remnants of activity in the biceps occurs simultaneously with the remaining shortened gaps of inactivity in the triceps. The reverse occurs during an increase of tension in the biceps. The

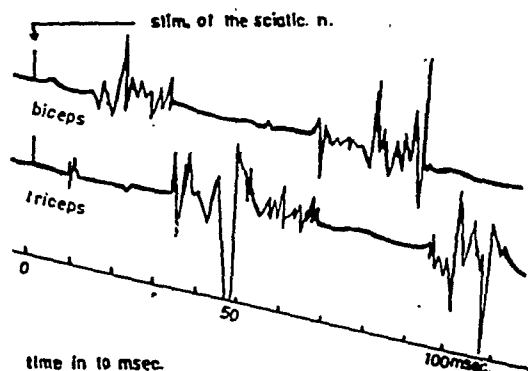


Fig. 1. Distribution of electrical mass activity from biceps and triceps (foreleg) following a sciatic shock on the ipsilateral side. Time in 10 msec.

experiments show that a single stimulus applied to the sciatic is followed by a periodical change of excitation in the cervical segments, and that, during the different phases of periodical spinal excitation, the facilitation of the extensor motoneurons is most intense when the inhibition of the flexor neurons is most profound.

2. Activity of single units in antagonistic muscles of the foreleg.

In order to study more carefully the rhythmical changes of reciprocal excitation, the spike activity of single units in the biceps and triceps muscles was recorded. In addition, the stimulus strength to the sciatic was kept so low that no muscle contractions were produced, but there was a definite influence on the rhythm of the myotatic discharges in the two antagonistic muscles. Records in Fig. 2 (from the biceps muscle of the foreleg) show the influence of subliminal, iterative, *contralateral sciatic shocks* on the rhythmical spike discharge (evoked by a slight constant tension in the muscle). Each sweep shows the events after each sciatic shock, the artefact of which is visible to the left on the records. A glance at the picture gives the impression that the spikes tend to occur within fairly well defined periods following stimulation.

Diagram 3 illustrates how the records from this and the following experiments have been analysed. In 3 B, thirteen different

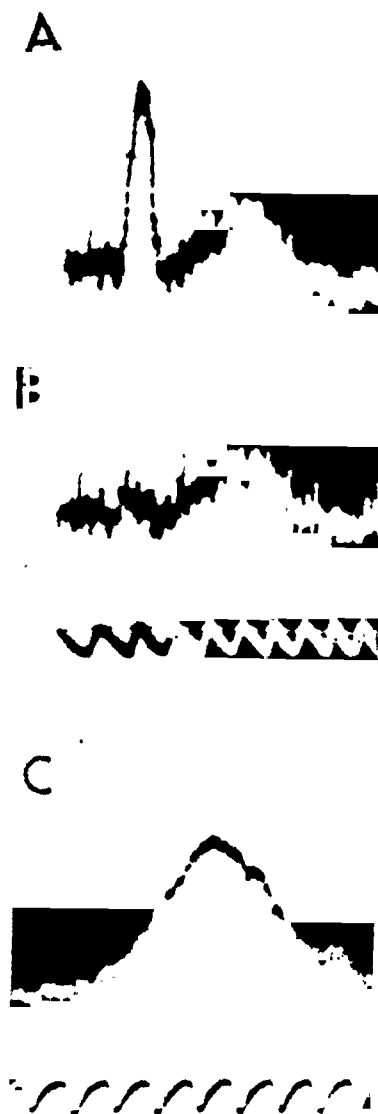


Fig. 2. Ventral root potentials from L_7 (left side). Medullary stimulus. Lumbo-sacral dorsal roots cut. A. Supra-threshold stimulus causing flexion of left hind leg. B. Sub-threshold stimulus. C. Same as B from another experiment, in which the potential from S_1 was measured. Time: 10 msec.

sweep pictures are arranged so that the interval between the sciatic shock (0 on the horizontal axis) and the first spike on each record increases from the top downwards in the diagram. As previously mentioned the changes of spinal excitability, following the sciatic shock, plays on a background of rhythmical activity maintained by the myotatic influence from the muscle during constant passive tension. In this case, the unit discharges with a frequency of 41 per sec. In 3 A the thick vertical lines represent the spikes of the rhythmically discharging unit prior to stimulation, the intervals representing the average for the intervals between the spikes (24.5 msec.).

The first spike in each of the upper two series in 3 B owing to short latency cannot be influenced by the central events following the sciatic shock. The second spike in each of the two upper tracings obviously is due to the myotatically conditioned rhythm since they occur 30 respectively 26 msec. after the first spike. Following this, one spike in the myotatically conditioned rhythm drops out; whereupon two spikes appear at typical intervals (24 respectively 20 msec.).

The following tracings show how the first spike on each sweep tends to be «caught» within the period 20—30 msec. after the stimulating shock, indicating a *first phase of facilitation*. Further, that spike which should appear about 25 msec. after the first one (*i.e.* about 50 msec. after the shock) drops out (3rd and 7th—13th tracings) or is delayed (4th—6th tracings). The disappearance or delay of the second spike indicates an *inhibition* of the motoneuron activity. During the period 70—130 msec., two spikes appear with about the same interval as for the regular rhythm in Fig. 3 A. Obviously a *second phase of facilitation* occurs during this period.

In diagram 3 C each column represents the number of spikes occurring within the periods 1—10, 11—20 msec, etc. after the stimulus (obtained from the tracings in 3 B). The diagram demonstrates the periodical changes in excitation of the flexor motoneurons during the first 150 msec. after the application of the contralateral sciatic shock.

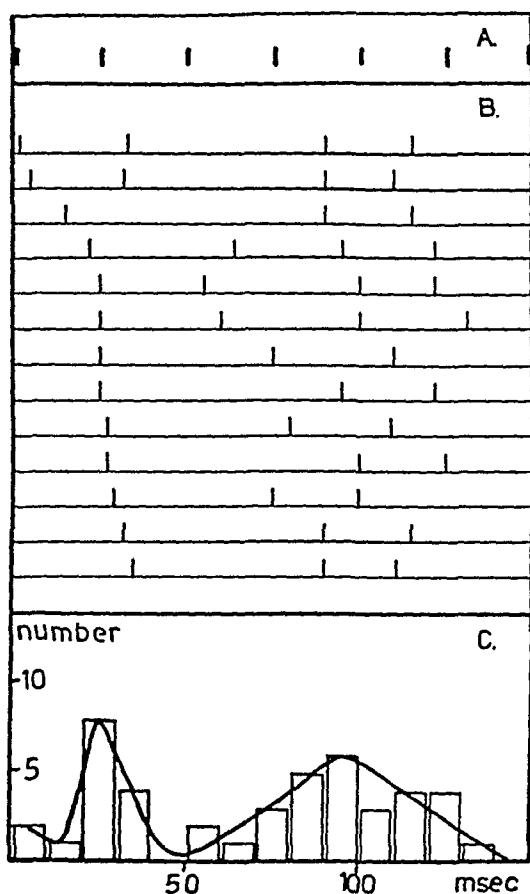


Fig. 3. Diagrams showing the time distribution of spikes from a single unit in biceps (left foreleg). A, average distribution prior to stimulation. B, thirteen sweep-pictures each one showing the distribution of the spikes after a shock to the right sciatic (0 in the horizontal axis). C, obtained from B; the columns represent number of spikes (vertical axis) within 0—10, 11—20 etc. msec. after the sciatic shock. (0 on the horizontal axis.)

The integrating curve indicates two periods of facilitation separated by a period of inhibition (cf. Fig. 1). Maximum facilitation occurs 20—30 msec. respectively 90—100 msec. after the stimulus.

It must be emphasized that the course of the curve obtained in the above experiment is dependent on *interference* between the myotatically evoked rhythm (in 3 A) and the periodic changes of excitability following sciatic stimulation. However, it is obvious, that the latter dominates.

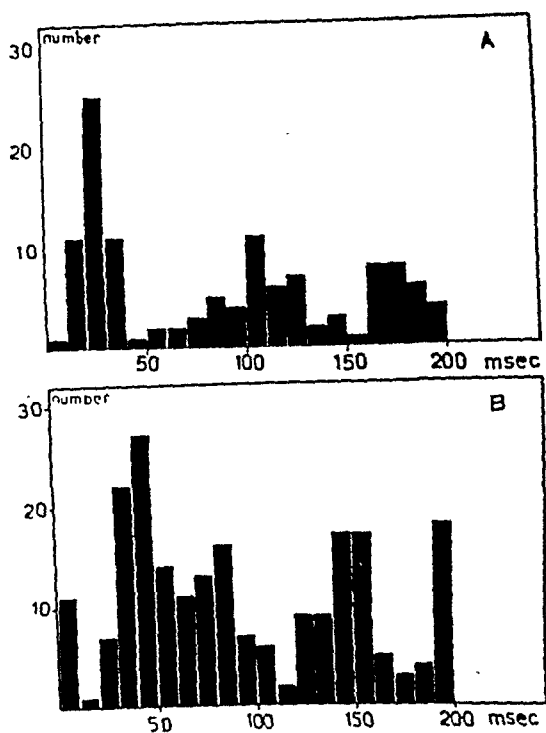


Fig. 4. Diagrams showing spike distribution of single units in biceps (A) and triceps (B) of left foreleg following a stimulus applied to the right sciatic nerve. Number of spikes on vertical axis. Time in 10 msec. on horizontal axis. (cf. Fig. 3.)

Several experiments were performed under similar conditions and the spikes from single units were recorded simultaneously from the triceps and biceps muscles of the foreleg. The stimulus was applied to either the contralateral or the ipsilateral sciatic nerve. Between 30 and 40 sweep records were used as basis for each diagram and the material was always arranged as shown in Fig. 3 C. Fig. 4 shows the typical distribution of the single spikes from units in the biceps (4 A) and the triceps (4 B) during the 200 msec. after a *contralateral sciatic shock*, which was too weak to evoke contraction of the foreleg muscles. The distribution of the spikes in Fig. 4 A is the same as in Fig. 3 with the addition of a third maximum of facilitation which occurs at about 180 msec. When examining the distribution of the spikes, obtained simultaneously from the antagonistic triceps muscle, it is obvious that the intensity of facilitation in this muscle is greatest when

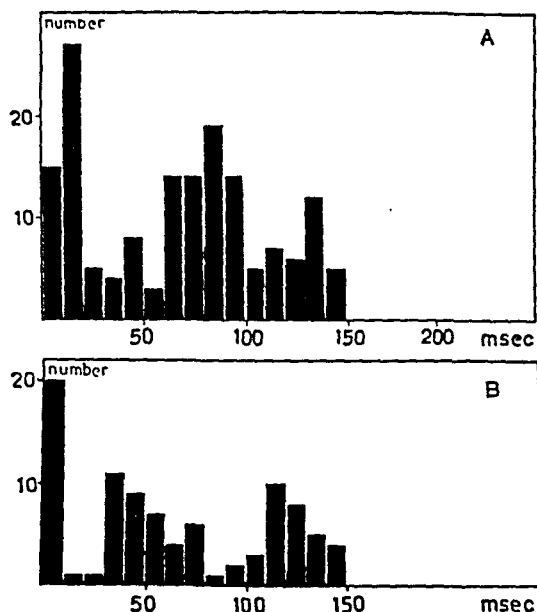


Fig. 5. Diagrams showing spike distribution of single units in biceps (A) and triceps (B) of left foreleg following a stimulus applied to the left sciatic nerve. Number of spikes on vertical axis. Time in 10 msec. on the horizontal axis. (cf. Fig. 3.)

there is a marked inhibition of the biceps activity (40—50 respectively 140—160 msec. after stimulus) and vice versa.

Irregularities in the distribution of values obviously depend on the ability of the myotatic rhythm to assert itself, see *e.g.* the first period of extensor facilitation (4 B) which includes two crests with an interval of 30—40 msec. (cf. BERNHARD and THERMAN 1947 c). Further it is apparent that the first phase, which is characterised by flexor facilitation and extensor inhibition (30—40 msec.), is of shorter duration than the following phases (70—100 msec.).

Fig. 5 from an experiment on the same pair of muscles, shows the distribution of the spikes from antagonistic units during 150 msec. following an *ipsilateral sciatic shock*. There is a narrow peak for the extensor at 10 msec. (column 0—10 msec. in 5 B) indicating raised excitation caused by the long ascending spinal reflex activity (cf. BERNHARD and THERMAN 1947 b; and for descending long spinal reflexes LLOYD 1942). The first maximum

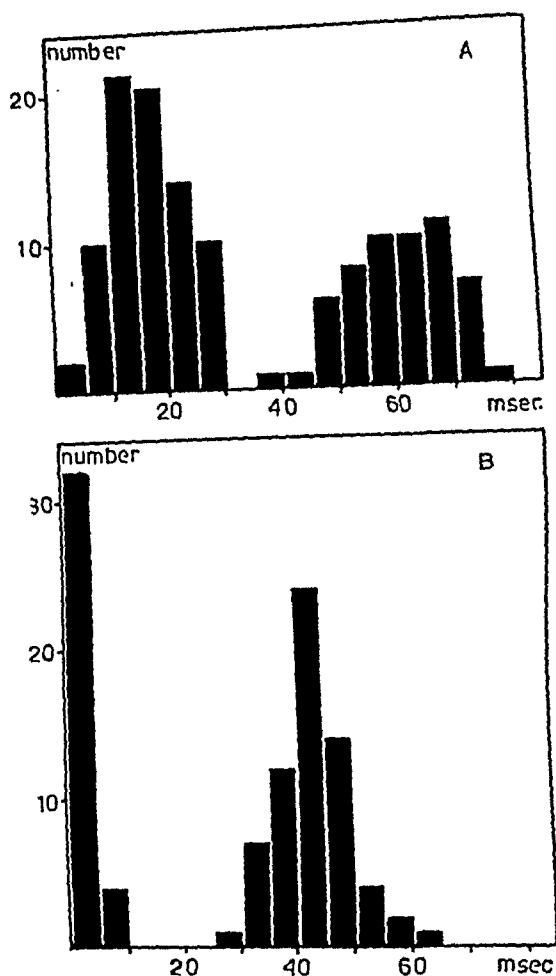


Fig. 6. Diagrams showing spike distribution of single units in right triceps (A) and left biceps (B) following stimulation of left radial nerve. Number of spikes on vertical axis. Time in 10 msec. on the horizontal axis. (cf. Fig. 3.)

of flexor facilitation occurs 20—30 msec. after the shock (5 A) corresponding to an inhibition of the extensor activity during the same period (5 B). The next two periods of extensor facilitation correspond fairly well to the periods of extensor inhibition (50 respectively 110 msec. after the stimulus).

Excitability curves have also been determined for motoneurons in the same antagonistic system after stimulation of the *radial nerve*. Fig. 6 shows the distribution during 80 msec. of the muscle spikes from units in the contralateral triceps (7 A) and the ipsilateral biceps (7 B) following a radial stimulus. The faster

sweep velocity in this case allowed more accurate measurements to be made and consequently each column in the diagrams represents 5 msec. The first column in 6 B, which represents an early period of facilitation, corresponds to the ipsilateral flexor reflex. After a period of inhibition a second phase of facilitation follows. In the contralateral triceps the facilitation is maximal at 10—20 respectively 60—70 msec., the intervening inhibition being greatest at 30—45 msec.

3. The influence of myotatic inhibition on the cycle of excitability.

The influence of changes in the proprioceptive in-flow on the rhythmical discharge from motor units under myotatic excitation has previously been analysed (BERNHARD and THERMAN 1947 c). In connection with the experiments above we studied the effect of changes in the myotatic excitation on the cyclic facilitation in cervical segments, following stimulation of the sciatic nerve. Record 7 *a* shows the usual distribution of spikes from a unit in the biceps of the foreleg when the ipsilateral sciatic is stimulated during liminal tension (cf. Fig. 5 A). In most of the pictures there is the typical double response of the single unit (cf. *e.g.* BERNHARD and THERMAN 1947 c). When the tension in the antagonistic triceps muscle is increased the latencies become longer (records 7 *b—d*) and at a certain tension the spikes drop out entirely. It should be noted that the latency for the first spike increases less than for the second, and also that the first is more »resistant» to the inhibitory influence evoked by the proprioceptive in-flow from the antagonist. This is graphically illustrated in Fig. 8 (latencies in msec. on the vertical axis and the number of sweep pictures on the horizontal axis). A slight increase of the tension in the antagonist (at A) is followed by a transitory prolongation of the second spike latency (open circles), while scarcely any change can be observed in the first spike latency (filled circles). When the tension is further increased (at B) there occurs a transitory prolongation of the second spike latency to about 150 msec., followed by a temporary disappearance

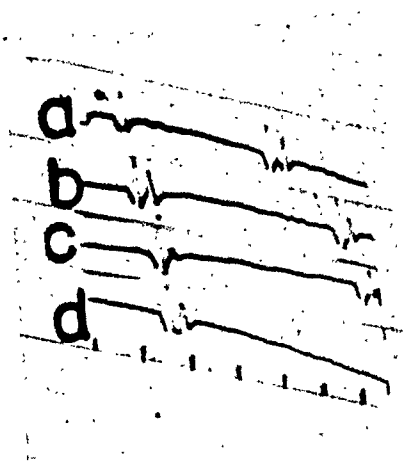


Fig. 7. Spikes from a single unit in left biceps following stimulation of the left sciatic nerve. *a*, during slight tension in biceps. *b*—*d*, during increasing tension in triceps. Time in 20 msec.

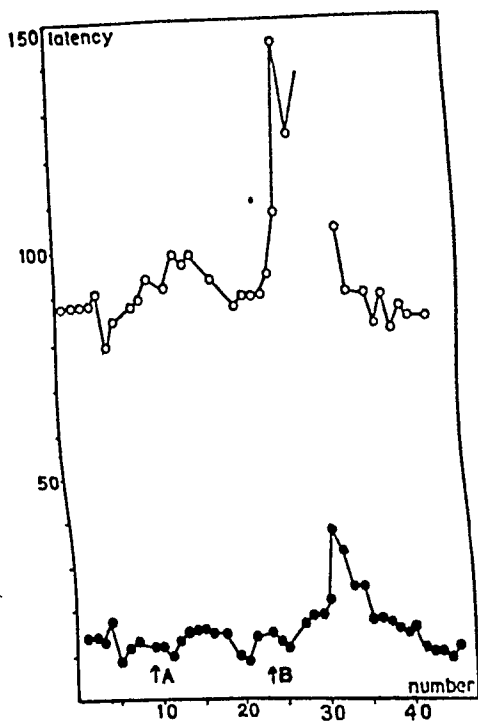


Fig. 8. Graphic illustration of an experiment similar to that in Fig. 7. Vertical axis gives the latencies of the first spike (filled circles) and the second spike (open circles). Number of recorded sweeps on horizontal axis. A, a slight increase and B, a further increase in tension of the antagonist.

of the spike discharge. The latency for the first spike is also temporarily prolonged but not to the same extent (from 15 to 40 msec.), nor does the first spike drop out.

4. Survey of the cyclic reciprocal facilitation and inhibition.

Fig. 9 gives a general view of the reciprocal facilitation and inhibition in biceps and triceps of the foreleg following stimulation of the sciatic (I), and the radial nerve (II) on the ipsilateral (A) and the contralateral side (B). Deflection *upwards* means facilitation of the extensor and inhibition of the flexor activity, whereas deflection *downwards* means facilitation of the flexor activity and inhibition of the extensor activity.

The latencies of the muscle spikes vary relatively less within the earlier phases of facilitation than in the later (see Fig. 3). As a consequence the early peaks in the different diagrams are in general narrower and higher than those occurring later in the cycle (Fig. 3, 4, 5 and 6). The relatively fixed latency indicates that the facilitation is more intense in the earlier phases than in the later. The fact that the first discharge is more resistant to inhibitory influences than the second (see Fig. 8) supports this assumption. Experiments of the type described on page 2 show that the same holds true concerning the periods of inhibition. Thus, the high amplitude of the early phases of the curves in Fig. 9 indicates that facilitation and inhibition are strongest at the beginning of the cycle.

After stimulation of the *sciatic nerve* the initial crest in Fig. 9 (I A) indicates facilitation of the extensor neurons and inhibition of the flexor neurons on the same side *i. e.* inclination to ipsilateral extension of the foreleg; whereas on the opposite side the first phase to occur is the initial dip in I B which indicates facilitation of the flexor neurons and inhibition of the extensor neurons *i. e.* inclination to contralateral flexion.

Stimulation of the *radial nerve* is followed by a phase characterised by facilitation of flexor neurons and inhibition of extensor neurons on the same side (II A), *i. e.* the ipsilateral flexor reflex;

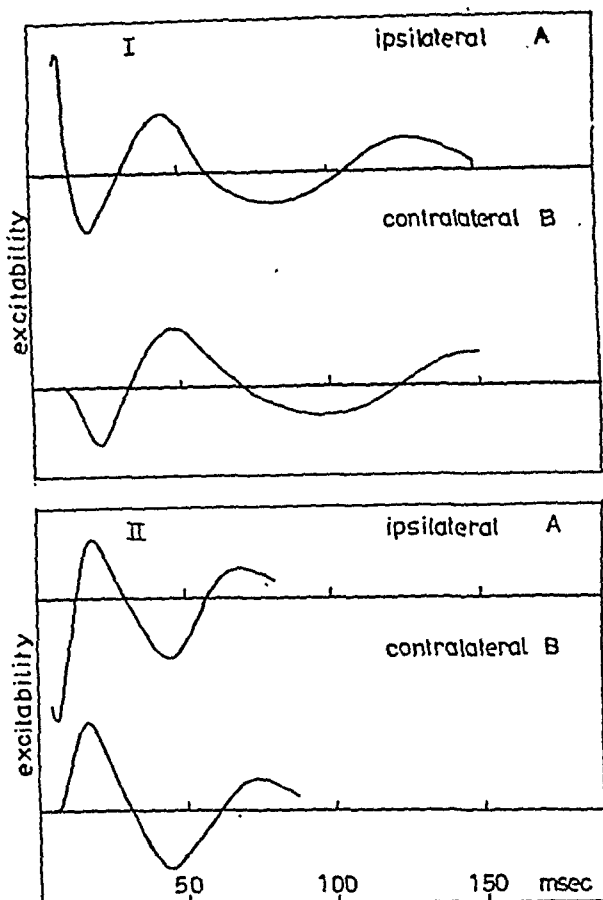


Fig. 9 Cyclic reciprocal changes of excitation in antagonistic motor systems (biceps and triceps of the foreleg) following stimulation of the sciatic (I) and radial (II) nerve on the ipsilateral (A) and contralateral side (B). For further description see text.

whereas the first peak in II B shows facilitation of the extensor neurons and inhibition of the flexor neurons on the opposite side *i.e.* inclination to a crossed extensor reflex.

5. Preliminary experiments on slow spinal cord potentials.

Having demonstrated the slow, cyclic, reciprocal effects after a single stimulus the question arose whether or not the different phases of excitation in the antagonistic systems could be correlated to different phases of the slow cord potential. Records

were made from the dorsal and ventral roots of lumbosacral segments when different afferent nerves of the hind leg were stimulated. We found that in the decerebrate state the ventral root potential following stimulation of *e.g.* the sural nerve may have a rhythmical shape, the different phases of which seem to have the same time characteristics as those of the cyclic excitability changes described above (see also preliminary note by BERNHARD and THERMAN 1947 a).

In connection with the question concerning the role of acetylcholine in central transmission and excitability (see *e.g.* SCHWEITZER and WRIGHT 1937 a and b, BÜLBRING and BURN 1941, FELDBERG 1945 and recently ECCLES 1947) the effect of this substance and also that of adrenaline was tentatively studied. Preliminary observations indicated that ACh may reduce the positive, whereas adrenaline may reduce the negative phase of the cyclic ventral root potential. This question has been further analysed by BERNHARD, SKOGLUND and THERMAN (1947).

Discussion

Certain reflections cannot be avoided when a comparison is made between our observations and the classical investigations on reflex activity.

Usually one single stimulus does not evoke any contralateral extensor reflex provided that the initial tension in the extensor is low (LIDDELL and SHERRINGTON 1923), whereas one shock easily elicits the ipsilateral flexor reflex (ECCLES and SHERRINGTON 1931), for which the central delay has been recently determined by LLOYD (1943). The first dip in diagram II A (Fig. 9) indicates the ipsilateral flexor reflex in the foreleg (cf. DENNY-BROWN and LIDDELL 1928) and the first crest in II B indicates that facilitation of the contralateral extension (cf. Fig. 6 A) occurs later and is less than that of ipsilateral flexion.

Further, stimulation of a hind foot is followed by increased extensor rigidity of the ipsilateral foreleg and flexion at the elbow of the contralateral foreleg (SHERRINGTON 1898 b). The initial effect in the excitability cycle of the antagonistic systems

of the foreleg following stimulation of the *sciatic nerve* is represented by the first narrow crest in I A (Fig. 9) indicating ipsilateral extension and reciprocal flexor inhibition. The initial dip in I B, representing contralateral flexor facilitation and reciprocal extensor inhibition, is delayed and of less amplitude.

There is a slight delay in the different phases of the excitability cycle on the contralateral side compared to that on the ipsilateral side (compare Fig. 4 A and 5 A). Except for this, the curves, representing the bilateral reciprocal excitability changes at the same spinal level, are fairly parallel regardless of whether an ipsilateral or a contralateral nerve has been stimulated (compare I A and B respectively II A and B in Fig. 9). Contrary to this, the different phases from the brachial level, following stimulation of a nerve of the foreleg, are opposite to those following stimulation of a nerve of the hind leg (compare I and II Fig. 9).

In classical investigations on the crossed extensor reflex no certain relationship between the rhythm of discharge in the muscle and the frequency of the stimulus could be found (FORBES and CATTELL 1924, COOPER and ADRIAN 1924, ADRIAN and BRONK 1929, DENNY-BROWN 1929). It should be pointed out that the typical grouping of activity, which we have found in the antagonists of the four legs, is dependent on a cyclic facilitation and inhibition of the motoneuron discharge maintained by myotatic excitation (see page 5). The rhythmical character of the excitability curves is not induced by proprioceptive volleys evoked by contraction in the muscles of the foreleg, since the stimulus strength is too low to give contractions in the muscles in question. Thus the cyclic changes of excitability must be centrally conditioned. In addition they may, however, be influenced by changes in the proprioceptive in-flow (see Fig. 7 and 8). It is interesting to note that an increase of inhibition induced from an antagonist is followed by an increased interval between the two first periods of facilitation.

The classical investigations by GRAHAM BROWN on the rhythmical reciprocal activity (1—2 per sec.) which he demonstrated to be centrally conditioned (see *e. g.* 1912) should be mentioned in connection with the above experiments. Our observations are also of interest in connection with the problem of »clonus». In

decerebrate cats this, »activity of an alternately self-exciting and self-inhibitory mechanism» (see CREED et. al. 1932), which can be evoked by tapping the tendon of an extensor has a rate of about 12 per sec. (see *e.g.* VIETS 1920, DENNY-BROWN 1929). The assumption that the rhythmical property of intraspinal structures may be of importance in the drive of clonus is favoured by similarity of the frequency of clonus and that of the spinal excitability changes (see Fig. 9). These changes are started by an afferent volley and are maintained by central processes.

The main purpose for this investigation has been to determine the characteristics of cyclic reciprocal excitability changes in order to find a starting-point on which to base further studies on reciprocal innervation (SHERRINGTON 1897, 1898 a). From the above observations and those previously described (BERNHARD and THERMAN 1947 c) two different questions became actual; 1) possible correlation between different phases of the spinal cord potential and those of the cyclic reciprocal excitation; 2) possible differences in the functional characteristics between flexor and extensor neurons. Both these problems have been attacked in preliminary experiments (see BERNHARD and THERMAN 1947 a); the first has been further analysed by BERNHARD (1947), and the second by THERMAN (personal communication).

Summary

1. A study has been made on the influence of sciatic and radial stimulation on the myotatically maintained rhythmical discharge of single units in antagonistic muscles (biceps and triceps) of the two forelegs.

2. It has been shown that a single stimulus applied to either one of the nerves evokes cyclic, reciprocal facilitation and inhibition of the extensor and flexor motor activity.

3. The cyclic changes of excitation are shown not to be governed by proprioceptive in-flow from the periphery.

4. The curve for the periodic changes of excitation at the same spinal level are fairly parallel following contra- and ipsilateral stimulation.

5. An inhibitory influence evoked by increased proprioceptive in-flow from the antagonist produces a delay in the different phases of facilitation. The later phases are more susceptible than the earlier ones.

6. Preliminary experiments on the correlation of cyclic reciprocal excitability changes and the features of slow spinal cord potentials are reported.

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Slow Cord Potentials of Opposite Sign correlated to Reciprocal Functions

By C. G. BERNHARD

Introduction

The present paper deals with the correlation between the positive and negative fractions of the ventral root potentials and the excitability changes in antagonistic reflex systems. It has been demonstrated (BERNHARD and THERMAN 1947 b and c) that a single stimulus applied to either one of the sciatic nerves evokes cyclic reciprocal facilitation and inhibition of the extensor and flexor motor activity. Preliminary experiments on the correlation of the cyclic changes of excitability and the features of the slow cord potentials recorded from the ventral roots were described. Interest was aroused by the tentative experiments to investigate the features of the slow cord potentials following stimulation of different nerves evoking reflex responses in antagonistic systems, and to test the antagonistic reflex activities during different phases of the slow potentials.

The experiments to be described show that reflex discharges, following afferent volleys in different nerves, are associated with slow polyphasic ventral root potential changes built up of positive and negative fractions, the degree of participation of each being dependent on the type of reflex activity. Further, the investigations show that antagonistic reflexes behave differently

during the potential phases of opposite sign. Some of the results have been recently reported in a short note BERNHARD and THERMAN 1947 a).

Methods

Decerebrate cats were used in all experiments. The left ventral roots L_6 , L_7 and S_1 were cut peripherally and either one of the L_7 or S_1 ventral root was placed on the recording electrodes (one electrode at the cut end and the other as near the cord as possible). The electrical stimuli to the different nerves (left and right sural nerves, left gastrocnemius and deep fibular nerves) were obtained from a stimulator, the shocks (0.3—0.5 msec. duration) being transmitted over transformers. The two first stages of the amplifier were direct coupled in push-pull and the last stages resistance-capacity coupled with a large time constant (2 sec.).

Results

1. Slow ventral root potentials following stimulation of the ipsilateral sural nerve.

Stimulation of the sural nerve is followed by ipsilateral flexion (SHERRINGTON 1910). It has been shown (LLOYD 1943 b) that the reflex discharge in the ventral root following stimulation of the lowest threshold fibres in the sural nerve has a central delay which indicates a multisynaptic transmission in the spinal cord (LLOYD's Group II reflex). The reflex discharge is not well synchronised but may be scattered throughout several waves during the first 8—10 msec. (see LLOYD 1943 b, BERNHARD 1945). In the experiments to be described below the main interest has been to study the slow potential changes occurring during the period about 100 msec. following the initial reflex discharge; consequently slow sweep velocities have been used. Thus, the pictures do not give the details concerning the latency and form of the initial discharge which is of less interest in this connection.

A



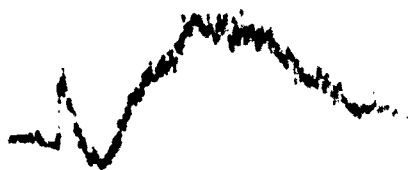
B



C



D



E

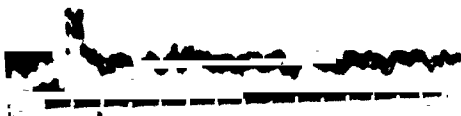


Fig. 1. A—E, ventral root potentials (L_7) following stimulation of the ipsilateral sural nerve, from five different experiments. Time in 10 msec.

The records in Fig. 1 show the slow potentials from the L₇ ventral root following stimulation of the ipsilateral sural nerve and are obtained from different experiments. Record 1 A shows the slow potential waves following the initial reflex discharge. As in the other records (except for 1 E) the picture is dominated by a prolonged negative wave, the maximum of which occurs at about 50 msec. after the beginning of the initial reflex discharge. This prolonged dominating negative wave is preceded by a slight positive dip the maximum of which occurs at about 15 msec. It must be emphasized that the amplitude of the different phases of the slow ventral root potential shows a considerable variation in different preparations. In records 1 A and C the positive overshooting below the base line is obvious. In record 1 B the potential fluctuation towards positivity does not cross the base line, whereas the negativity is well pronounced and of longer duration than in records 1 A and C. Record 1 D shows a positive dip followed by an enormous negativity, the amplitude of which is about twice that of the reflex discharge. Finally, record 1 E is from a preparation which did not give any visible slow potentials with the time characteristics described above. It may be pointed out that record 1 E was obtained from a preparation in good condition in which excellent reflex volleys could be elicited (note the pronounced initial multisynaptic reflex discharge).

Thus, the ability to develop the slow ventral root potentials described above varies considerably from case to case. It is obvious that the state of spinal excitation (dependent on the level of transection and the afferent in-flow) is of great importance. It is also clear that the factors which govern these slow potentials of long duration are independent of those which determine the capacity of the spinal cord to transmit reflex discharges evoked by synchronised afferent volleys (cf. records 1 D and E). In preparations with well pronounced slow potentials positivity as well as negativity is evident at a stimulus strength of 3—5 times the threshold value for the multisynaptic reflex. An increase of the stimulus strength above 10 times the threshold value does not evoke any further increase of the slow potentials. It has also been shown that the amplitude of the slow ventral root potentials is

greater the nearer the proximal electrode is to the spinal cord. It can further be stated that the picture is always dominated by slow negativity with a maximum at about 50 msec. (30—70 msec.) and a duration of about 100 msec. (60—140 msec.). In cases with well pronounced slow potentials the amplitude of the negative wave is about 0.1 mV. The preceding dip towards positivity, the amplitude of which varies independently of the negativity, is maximal about 15 (12—20) msec. after the beginning of the reflex discharge. It should be emphasized that the S_1 , L_7 and usually the L_6 ventral roots are cut so that muscle contractions in the hind leg are prevented.

2. Excitability changes following multisynaptic activity elicited from the ipsilateral sural nerve.

Stimulation of the afferent fibres of lowest threshold in the gastrocnemius nerves is followed by a well synchronised reflex potential in the ventral root. The short central delay of the ventral root discharge indicates that the reflex is mediated through a monosynaptic reflex arc (Group I reflex, LLOYD 1943 a and b). This discharge reflects into the muscle, the large afferents of which are stimulated (LLOYD 1943 b and c). Consequently the reflex spike represents activity in the extensor motoneurons.

Records in Fig. 2 show the excitability changes of the *monosynaptic extensor reflex* preceded by a multisynaptic flexor reflex. The interval between the conditioning shock applied to the sural nerve and the test shock applied to the gastrocnemius nerves increases downwards in the series of records (2 A—K). Record 2 L shows the magnitude of the monosynaptic extensor reflex following the unconditioned test shock. In 2 A the monosynaptic test response occurs during the decrement of the multisynaptic discharge. When the interval between the conditioning shock and the test shock is increased the monosynaptic response is first inhibited (2 B and C; interval 10 and 15 msec. respectively). At greater intervals it becomes larger (2 D) and at still greater intervals (2 E—F) the amplitude of the monosynaptic response is

A

B

D

G

J

K

L

Fig. 2. A—K, conditioning of a monosynaptic reflex (evoked from the gastrocnemius nerves) by a preceding multisynaptic response (evoked from the ipsilateral sural nerve). Time interval between the conditioning response and test response increase downwards in the series. L, unconditioned test response. Time in 10 msec.

much higher than that of the unconditioned response (cf. 2 L). When the interval is further increased the amplitude is reduced (2 H—K). Thus, the multisynaptic reflex evoked from the sural nerve is followed by slow intraspinal processes the different phases of which have different influences on the excitability of the monosynaptic extensor reflex. During the phase following 10—20 msec. after the multisynaptic response the monosynaptic extensor reflex is reduced. During the second phase the monosynaptic extensor reflex is increased, the facilitation being most pronounced about 50 msec. after the multisynaptic discharge.

Figs. 3 A—C and 4 A and B show the correlation between the different phases of the slow ventral root potential following stimulation of the sural nerve and the excitability changes in the monosynaptic extensor reflex preceded by a multisynaptic reflex elicited from the sural nerve. In diagrams 3 A and 4 A the ventral root potential is given in per cent of the amplitude of the initial multisynaptic response. In diagrams 3 B—C and 4 B the amplitude of the conditioned test response is plotted (in per cent of the unconditioned response) against the interval between the conditioning response and the test response. The diagrams show that the inhibition of the monosynaptic extensor-reflex corresponds to the dip towards positivity following the multisynaptic reflex (3 and 4 A); whereas the following facilitation of the monosynaptic response corresponds fairly well to the long-lasting negative potential wave.

In the experiments illustrated in 3 B and 4 B the monosynaptic test reflex has been kept near the threshold. The two diagrams demonstrate the variation of the facilitation corresponding to the late negative phase (about 200 per cent in 3 B and about 1000 in 4 B). A systematic correlation between the amplitude of the negative wave and the magnitude of facilitation in different experiments has not been made. It is obvious, however, that it is impossible to obtain any excitability changes of the kind demonstrated above in those cases where stimulation of the sural nerve is not followed by slow ventral root potentials despite a pronounced reflex discharge (see Fig. 1 E).

In diagram 3 C the amplitude of the monosynaptic test discharge was about 25 times that in diagram 3 B owing to a

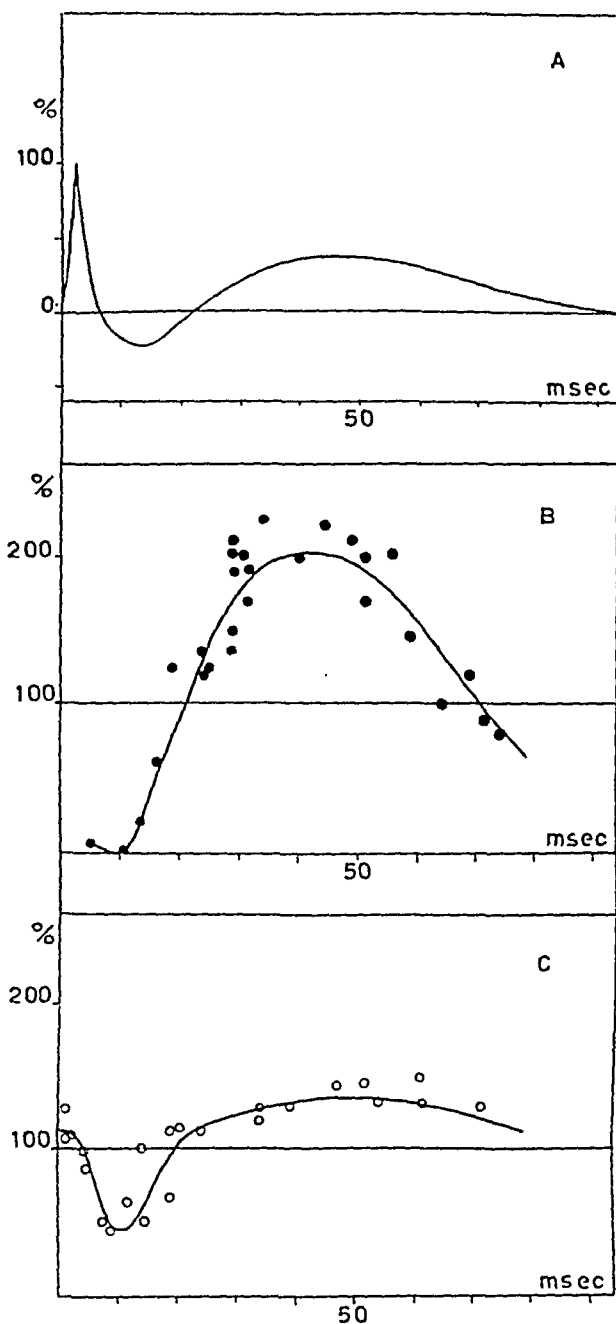


Fig. 3. A, ventral root potential (S₁) following stimulation of the ipsilateral sural nerve, plotted in per cent of the amplitude of the reflex discharge. B—C, influence of a preceding multisynaptic reflex (same as in A) on a following monosynaptic reflex (evoked from the gastrocnemius nerves). Amplitude of monosynaptic response in per cent of unconditioned response plotted against intervals between the conditioning response and test response, the latter being slight supraliminal in B and slight submaximal in C.

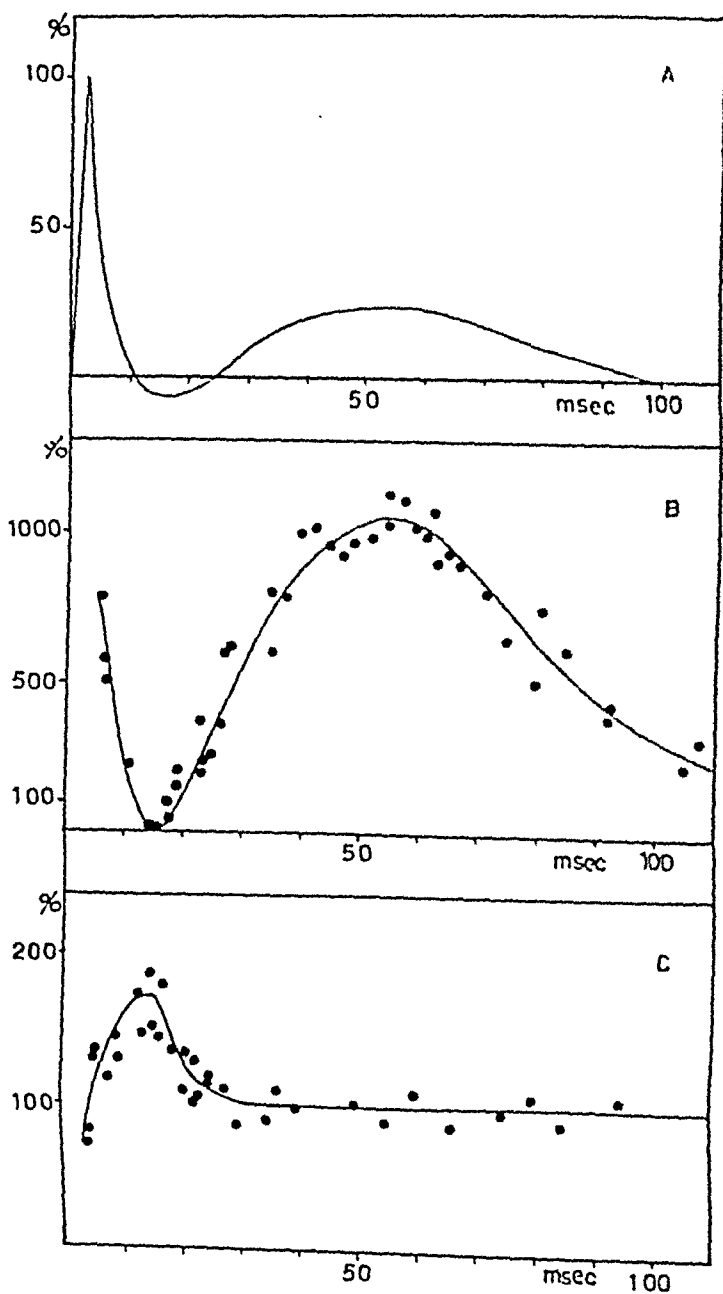


Fig. 4. A, ventral root potential (L_7) following stimulation of the ipsilateral sural nerve (in per cent of amplitude of reflex discharge). B—C, influence of a preceding multisynaptic reflex (same as in A) on a following monosynaptic reflex evoked from the gastrocnemius nerves (B), and on a monosynaptic reflex evoked from the deep fibular nerve (C).

stronger stimulus to the gastrocnemius nerves. The inhibition corresponding to the positive dip is well pronounced. As is to be expected the late facilitation cannot assert itself to the same extent as in diagram 3 B and 4 B, based on test effects of lower amplitude.

A *monosynaptic flexor reflex* (LLOYD 1946 a and b) has also been tested during the period of 100 msec. following after the multisynaptic reflex evoked from the sural nerve. In these experiments the test shock, applied to the deep fibular nerve, was kept just above the threshold for the monosynaptic response. It should, however, be pointed out that the monosynaptic flexor reflex is often difficult to obtain in decerebrate preparations with pronounced extensor rigidity. Diagram 4 C is from the same experiment as 4 A and B and shows the behaviour of the monosynaptic flexor reflex at different intervals following the multisynaptic reflex. Since the strength of the conditioning stimulus applied to the sural nerve was the same as in diagram 4 B a comparison of the three diagrams can be made. Diagram 4 C shows that the monosynaptic flexor reflex is facilitated within the period corresponding to the positive dip of the ventral root potential (4 A), *i. e.* the period during which the monosynaptic extensor reflex is inhibited (4 B). During the late phase of slow negativity (characterised by facilitation of the monosynaptic extensor reflex) any influence on the monosynaptic flexor reflex could not be found. The facilitation of the monosynaptic flexor response within the period 10—20 msec. after the multisynaptic response varies considerably depending on the state of excitation in different preparations, strength of stimulus etc. In some experiments it was found to be about 700 per cent.

Finally, the influence of a preceding *multisynaptic reflex* elicited from the sural nerve on the reflex evoked from the same nerve was studied. Fig. 5 shows the ventral root potential (L_7) following stimulation of the sural nerve (VP; in per cent of maximal reflex discharge), the changes of the multisynaptic reflex discharge evoked from the same nerve (open circles) and the changes of excitability in the monosynaptic extensor reflex (filled circles) during a period of 100 msec. following the conditioning

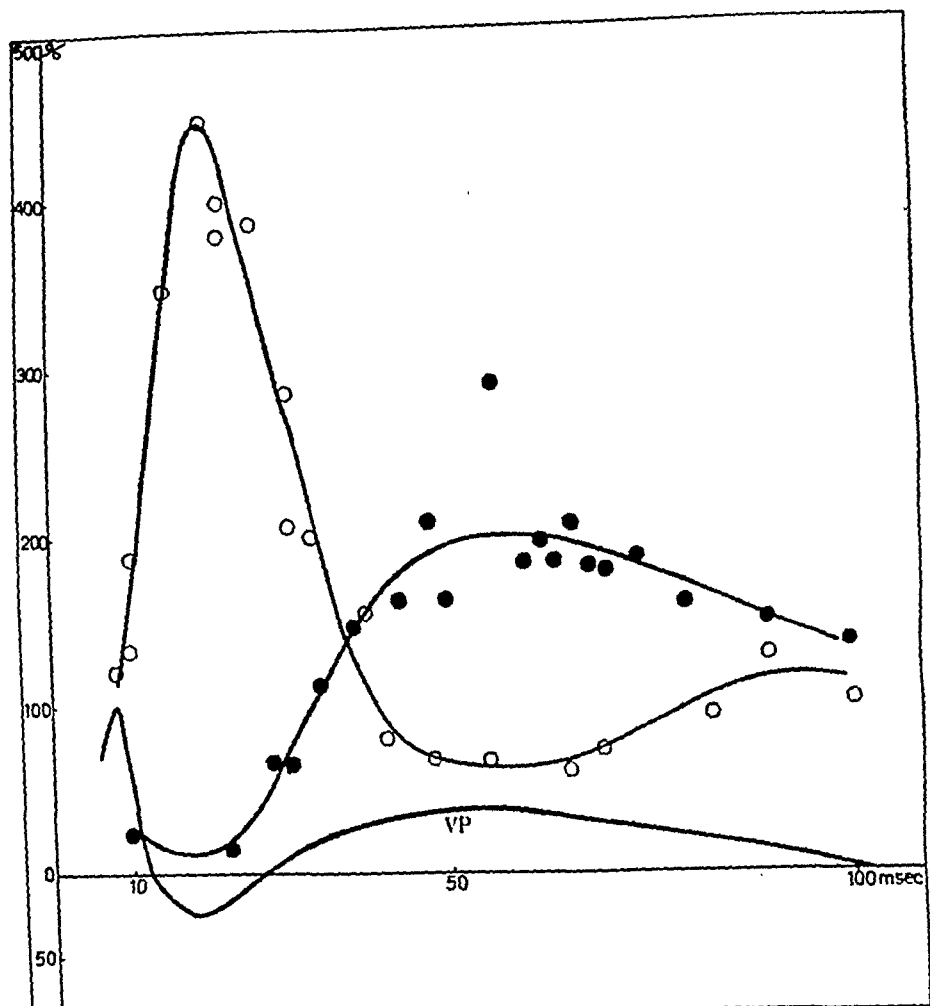


Fig. 5. VP, ventral root potential (L_7) following stimulation of the ipsilateral sural nerve. Facilitation and inhibition of a monosynaptic reflex elicited from the gastrocnemius nerves (filled circles) and of a multisynaptic reflex response elicited from the sural nerve (open circles) during the different phases of the slow ventral root potential following stimulation of the sural nerve.

sural stimulus. The excitability curve for the monosynaptic extensor response has the same course as was demonstrated in Figs. 3 and 4 with a period of inhibition corresponding to the positive dip of the ventral root potential and a period of facilitation during the late slow negativity. On the contrary the facilitation of the multisynaptic reflex evoked from the sural nerve

coincides with the positive dip of the slow ventral root potential and there is an insignificant inhibition during the late negative phase.

3. Slow ventral root potentials and excitability changes following stimulation of the contralateral sural nerve.

The slow ventral root potential (from L_7 or S_1) is still more variable following stimulation of the contralateral sural nerve. It must be kept in mind that a single crossed stimulus often is subliminal but may become liminal if the central excitation is raised, a fact which is known from classical investigations. Thus, an extensor reflex may be evoked by a single crossed stimulus when the proprioceptive in-flow is augmented by increasing the initial tension in the extensor muscle (see CREED *et al.* 1932). Further, the level of transection is of great importance since the reflex action is to a great extent dependent on influences from supraspinal levels. When the motoneurons are subjected to a sufficient background excitation — of central and peripheral origin — a multisynaptically transmitted reflex response can be led off from the ventral root (L_7) following stimulation of the contralateral sural nerve. In these cases the crossed reflex response is usually followed by a slow ventral root potential the features of which are illustrated in Fig. 6. The asynchronous discharge of low amplitude is followed by a dominating positivity with a maximum 30—40 msec. after the beginning of the discharge (amplitude about 0.07 m V). On the slope towards positivity there is a crest of negativity with a maximum 10—20 msec. after the beginning of the reflex discharge. After about 70 msec. the potential usually becomes negative again.

The excitability of the *monosynaptic extensor reflex* has been studied during the period corresponding to these slow potential changes. Thus, the conditioning shock was applied to the sural nerve on one side, and the test shock was delivered to the gastrocnemius nerves on the other side. The reflex activity was led off from the S_1 ventral root on the same side as that of the

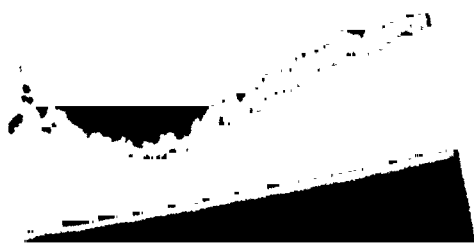


Fig. 6. Ventral root potential (I_r) following stimulation of the contralateral sural nerve. Time in 10 msec.

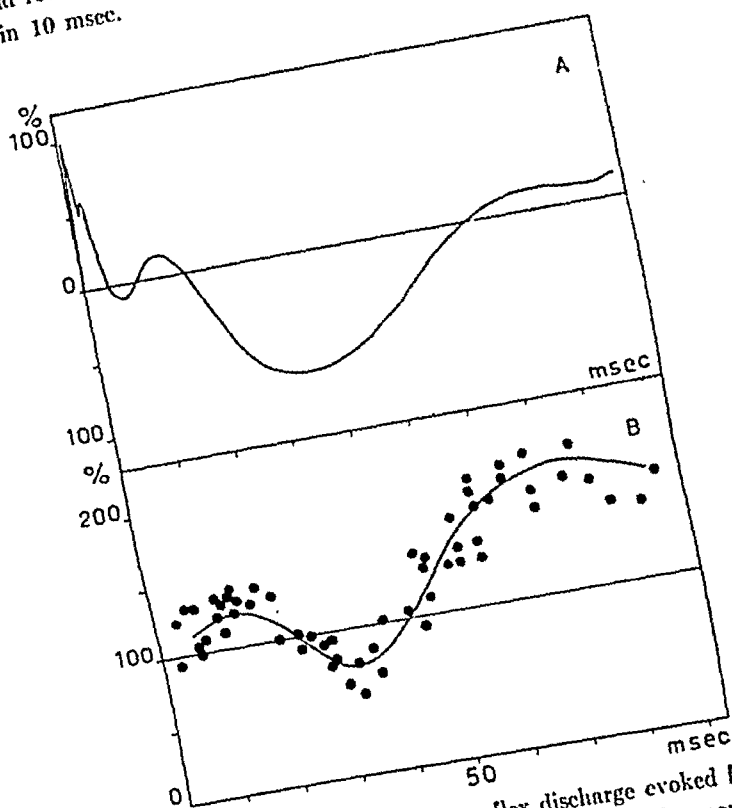


Fig. 7. A, ventral root potential following reflex discharge evoked from the contralateral sural nerve (cf. Fig. 6). B, conditioning effects on the monosynaptic reflex (evoked from the gastrocnemius nerves) during the different phases following a contralaterally evoked reflex response (same as in A).

test stimulus. Diagram 7 B shows the excitability curve for the monosynaptic extensor reflex from such an experiment. The amplitude of the monosynaptic spike in per cent of unconditioned test spike is plotted against the interval between the crossed reflex discharge and the test spike. The excitability cycle begins with facilitation of the monosynaptic extensor reflex. This is, however, broken down by an inhibitory process, which brings the curve below 100 per cent. The inhibitory action is maximal 30—40 msec. after the conditioning crossed reflex response (70—80 per cent). After the dip the facilitation again becomes predominant being maximal (180 per cent) about 70 msec. after the conditioning reflex response.

The crest indicating a slight facilitation (at 10—20 msec.) corresponds to the negative wave on the slope towards positivity of the slow ventral root potential following the conditioning reflex discharge (in Fig. 7 A) and the period of inhibition in 7 B corresponds fairly well to the positive dip in 7 A, the lowest point of the curve in both cases occurring between 30 and 40 msec. The following increase of excitation corresponds to the rising phase of the slow ventral root potential (from 35—70 msec.).

In many preparations the contralateral reflex to one single shock may be impossible to elicit, the background excitation being too weak. Fig. 8 A shows the features of the ventral root potential (L_7) following stimulation of the contralateral sural nerve which failed to elicit any reflex discharge. The central events following the contralateral afferent volley is accompanied by an insignificant positive dip succeeded by a long-lasting negative wave.

The *monosynaptic extensor* reflex was tested during the different phases of the slow potential following the contralateral conditioning stimulus. (Fig. 8 B). A period of inhibition is followed by a long-lasting period of facilitation which coincides with the small negative wave in 8 A; both being maximal about 40 msec. after the application of the conditioning stimulus. In this case a *multisynaptic reflex* following stimulation of the sural nerve (ipsilateral flexor reflex) was also tested, the conditioning stimulus to the contralateral sural nerve being the same as in

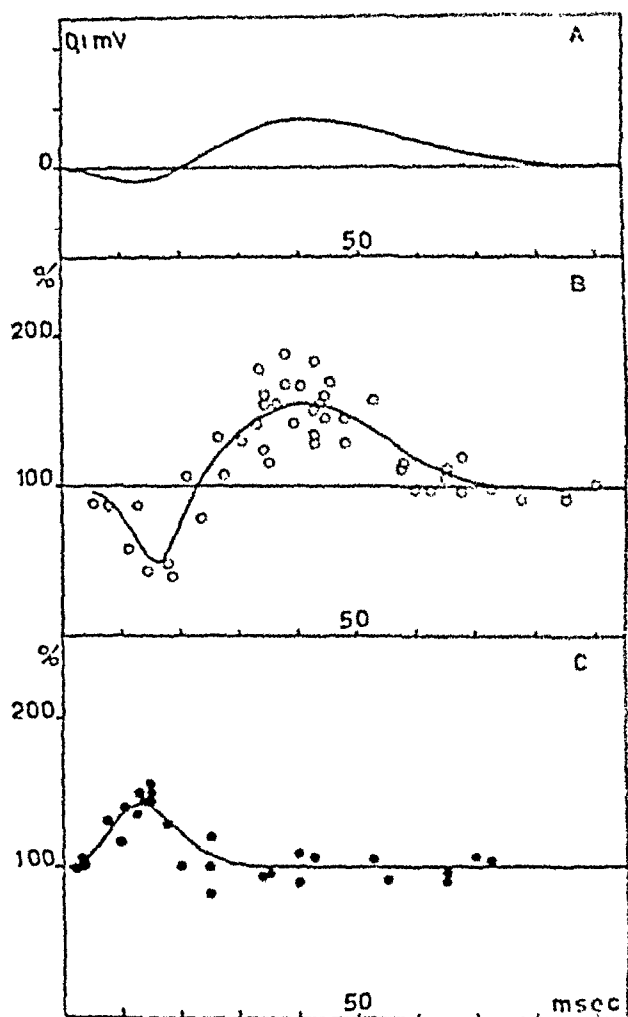


Fig. 8. A, features of ventral root potential following an afferent volley in the contralateral sural nerve which does not evoke any reflex discharge.

B—C, excitability changes of the monosynaptic response evoked from the gastrocnemius nerves (B) and on the multisynaptic flexor response evoked from the sural nerve (C) during the different phases of the slow potential following the contralateral stimulus (Same as in A).

8 B. Fig. 8 C shows that the excitability curve for the multisynaptic ipsilateral flexor reflex has an entirely different course. It is characterised by a period of facilitation corresponding to the inhibition of the monosynaptic extensor reflex and the insignificant positive dip of the ventral root potential.

4. Slow ventral root potentials and excitability changes following stimulation of the ipsilateral gastrocnemius nerves.

As already mentioned stimulation of the low threshold afferent fibres of the gastrocnemius nerves evokes a monosynaptically transmitted reflex discharge in the S_1 ventral root. The monosynaptic reflex response following stimulation of the muscle afferents of lowest threshold is not followed by any measurable slow ventral root potential. When, however, the stimulus strength is increased the monosynaptic spike in the ventral root is followed by a short negativity succeeded by a slight long-lasting positive potential which is illustrated in Fig. 9. The monosynaptic reflex spike which cannot be seen in the records reaches far above each picture. The following decrement of the negativity is clearly visible as well as the smooth long-lasting positive wave, which is best developed in record 9 A and has a duration of about 100 msec. (70—120 msec.). The slow positive wave usually becomes

A



B



Fig. 9. Slow ventral root potential (S_1) following stimulation of the ipsilateral gastrocnemius nerves. A and B from two different experiments. Time in 10 msec.

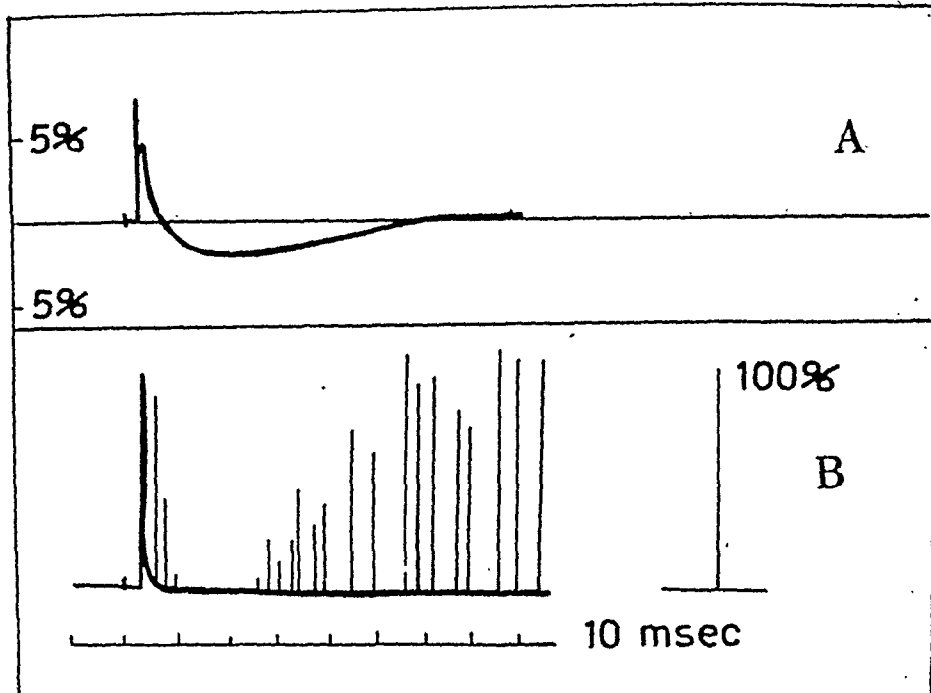


Fig. 10. A, slow ventral root potential in S_1 , following stimulation of the ipsilateral gastrocnemius nerves (in per cent of monosynaptic spike amplitude).

B, excitability changes following a monosynaptic reflex response; both conditioning (thick line) and test responses (thin lines) elicited from the gastrocnemius nerves. Unconditioned test response to the right in B (100 %).

visible when the strength of the stimulus applied to the gastrocnemius nerves reaches a value about 10 times the threshold value for the monosynaptic response. The amplitude is much less than for the slow potentials following stimulation of the sural nerve. Usually it amounts to about 3–10 % of the monosynaptic reflex spike *i. e.* 20–40 microvolts only. Like the slow potentials following a sural volley the magnitude of the slow positive wave following stimulation of the gastrocnemius nerves varies in relation to the reflex spike in different preparations. When the stimulus strength to the gastrocnemius nerves is further increased the shape of the slow ventral root potential does not change *i. e.* any late negativity similar to that following stimulation of the sural nerve never occurs.

Fig. 10 A shows the slow ventral root potential following a monosynaptic reflex response (the initial phase is indicated in the diagram) evoked by stimulation of the gastrocnemius nerves. In diagram 10 B the excitability changes in the same monosynaptic arc are illustrated, the conditioning as well as the testing shocks being applied to the gastrocnemius nerves. The average value for the unconditioned test effect is to be found to the right in Fig. 10 B. As can be observed the stimulus strength of the conditioning and testing shocks was the same giving spikes of similar magnitude. The diagram shows the reduction of the second monosynaptic response (indicated by thin vertical lines) during a period approximately corresponding to the duration of the positive ventral root potential in Fig. 10 A. The test effect is completely abolished during the time interval 7—22 msec. after the conditioning discharge. The same excitability curve was obtained when the conditioning shock was applied to one and the test shock to the other of the nerves to the gastrocnemius muscle.

Discussion

An analysis of the slow potentials in the spinal cord were first made in the classical paper by GASSER and GRAHAM (1933). On the basis of these and later investigations (HUGHES and GASSER 1934 a and b; HUGHES, MCCOUCH and STEWART 1937) the slow negative and positive potentials, led off from the surface of the cord, following afferent stimulation were referred to internuncial neurons (see also MCCOUCH *et al.* 1941, 1943).

Slow negative potentials in the dorsal roots of the spinal cord were first observed by GOTCH and HORSLEY (1891). Later many investigations have been performed on the cord potentials using ventral or dorsal roots as leads (UMRATH 1933, UMRATH and UMRATH 1934, BARRON and MATTHEWS 1936 a and b, 1938 a and b, BONNET and BREMER 1938 a and b, FESSARD and MATTHEWS 1939, DUN 1941, 1942, BREMER *et al.* 1942, DUN and FENG 1944, ECCLES 1946, ECCLES and MALCOLM 1946). Using LLOYD's technique (1943 a and b) for the production of a monosynaptic reflex discharge ECCLES (1946) investigated the

initial catelectrotonic ventral root potential (frogs and cats), evoked without complications produced by internuncial activity. This initial ventral root negativity which may be regarded as a fraction of the negativity evoked by stimulation of different nerves or dorsal roots (BARRON and MATTHEWS 1938 a) is referred by ECCLES to the motoneurons and regarded by him as an impulse generating potential (the synaptic potential) homologous to the similar potential at the sympathetic ganglion synapses (ECCLES 1944) and at the endplate (ECCLES, KATZ and KUFFLER 1941).

The slow ventral root positivity observed (in frogs) by UMRATH (1933) has been questioned (BARRON and MATTHEWS 1938). ECCLES, however, described a slight »after-positivity« in the ventral roots (in cats) following the synaptic potential in the monosynaptic reflex arc which begins 10—15 msec. after stimulation of the afferent nerve (1946).

In the present paper it has been shown that, dependent on the central state of excitation, the reflex response following stimulation of different nerves in decerebrate cats is accompanied by polyphasic ventral root potentials characterised by negative as well as positive phases. The investigations deal with the slow potential changes occurring about 10 msec. after the reflex discharge and displaying their action during 100 msec. Interneuron activity is not excluded since exteroceptive nerves (sural nerves) have been stimulated and relatively strong stimuli to the muscle afferents have been used. The principle objective was, however, to investigate whether or not activity in antagonistic reflex systems is accompanied by slow potential changes of different shape in the ventral roots, and to find a correlation between the excitability changes in the antagonistic reflex systems and the different phases of the slow potentials. Obviously afferent volleys giving reflex discharges in the ventral roots evoke intraspinal processes for which the negative and positive fractions of the slow potentials in the ventral roots are significant expressions. The above experiments, indicate that, when afferent stimuli evoking flexor reflex responses are used (Fig. 1, 3, 4, 5), the negative fraction is dominant, while stimuli evoking extensor reflex activity are associated with dominant positivity (Fig. 6, 7, 9 and

10). The slow potentials are not to be regarded as after-potentials in the extraspinal part of the motoneurons, since they diminish with increasing distance from the spinal cord and may occur without preceding impulse discharge (Fig. 8). Their origin may be of pre-synaptic order (*e. g.* interneurons; cf. GASSER and GRAHAM 1933, HUGHES and GASSER 1934 a and b concerning slow potentials of similar time constants recorded from the surface of the cord) or they may be produced in the different intraspinal structures of the motoneurons (cell bodies or dendrites). In any case the facts regarding antagonistic reflex responses and potential sign may indicate differences in the organisation of the intraspinal motoneuron structures of the antagonistic systems including their specific orientation to each other and their relationship to the presynaptic elements. Further, it has been shown that the antagonistic reflexes behave differently during the periods of negativity and positivity. It should be emphasized, that flexor and extensor systems are selectively activated when artificial stimuli of opposite direction are applied to the cat's spinal cord (SKOGLUND 1946, 1947). It is possible that both observations indicate a different organisation of the intraspinal structures of the antagonistic motoneurons. Experiments for the elucidation of this question are extremely necessary and in fact such investigations have already been started. A preliminary report (ÅSTRÖM 1947) gives some indication that there may be a different orientation of the motoneurons belonging to antagonistic systems.

In this connection an interesting statement made by CAJAL (1909) is very suggestive. On the basis of histological observations CAJAL stressed that, in reflex arcs which serve the monosynaptic impulse transmission the dorsal collaterals terminate on the cell-bodies of the motoneurons, while in reflex arcs mediating multisynaptic reflexes, the intervening interneurons terminate on the huge dendritic branchery of the motoneurons. It should be pointed out that those reflex responses which are transmitted in reflex arcs belonging to the latter group are associated with pronounced slow potentials (see Figs. 1 and 6). Thus there is a possibility that the engagement of the rich motoneuron dendritic network gives rise to the large slow potentials. If the intraspinal structures of the motoneurons are the origin for the

slow potential changes then the rôle played by the dendrites must be important. The slow potential changes demonstrated above may then be compared with the R_3 deflections *i. e.* changes of the L fraction of the membrane potential (LORENTE DE NÓ 1947). There are many indications in support of the above assumptions which will be discussed in connection with later investigations. At present mention will only be made of the fact that the spread along the motoneurons is characteristic for the L fraction changes and that »any change in the value of the L fraction causes a change in the excitability of the nerve fibres» (LORENTE DE NÓ 1946).

Finally, it should also be kept in mind that there seems to be differences in the characteristics of flexor and extensor motoneurons (BERNHARD and THERMAN 1947; THERMAN, personal communication).

Summary

1. In experiments on decerebrate cats records have been made of the slow ventral root potentials (from L_7 and S_1) following stimulation of different afferent nerves. Monosynaptic and multisynaptic (extensor and flexor) reflexes have been tested during different phases of the slow potential changes.

2. It has been shown that the reflex response following stimulation of different nerves of the hind legs may be accompanied by polyphasic slow ventral root potentials which are decrementally transmitted along the roots. The amplitudes of the positive and negative fractions contributing to the polyphasic potential depend on the source of the afferent volley. The different fractions show considerable variations due to differences in the general state of central excitation for which the transection level and intensity of proprioceptive in-flow is of great importance. The development of the slow potentials is independent of the ability of the spinal cord to transmit reflex responses evoked by synchronised afferent volleys.

The following observations apply to experiments in which well pronounced slow potentials were obtained.

3. *a.* The multisynaptic reflex response (L_7 and S_1 ventral root) following stimulation of the *ipsilateral sural nerve* (ipsilateral flexor reflex discharge) is always followed by a dominant slow negative wave (maximum about 50 msec. after the reflex discharge; duration about 100 msec.). The slow negativity is generally preceded by a positive dip (maximum 15—20 msec. after the reflex response) which separates the initial negative decrement from the late negative wave.

b. The slow ventral root potential (L_7 and S_1) following stimulation of the *contralateral sural nerve* depends on whether a reflex discharge is evoked or not. If a reflex discharge (contralateral extensor reflex) is evoked it is generally followed by a dominant positive wave of long duration (70—100 msec.). A slight negative crest may occur 10—20 msec. after the reflex response. (If reflex response does not occur the contralateral effect may only be a long negative wave preceded by an insignificant positive dip).

c. The maximal monosynaptic reflex spike following stimulation of the *ipsilateral gastrocnemius nerves* (monosynaptic extensor reflex) is accompanied by a dominant slight positivity (duration about 100 msec.). An increase of the stimulus strength never evokes any late negative wave.

Thus, afferent volleys (ipsilateral sural nerve) evoking multisynaptically transmitted reflex responses in the flexor motoneurons are accompanied by a dominant negativity, while afferent volleys (contralateral sural nerve and ipsilateral gastrocnemius nerve) eliciting multi- and monosynaptically transmitted activity in the extensor motoneurons are accompanied by a dominant positivity.

4. Characteristic changes in reflex excitability are demonstrated during the different phases of the slow ventral root potentials.

The monosynaptic reflex discharge (in S_1 ventral root) following stimulation of the *gastrocnemius nerves* (monosynaptic extensor reflex) is always facilitated during periods when the negative fraction of the polyphasic potential is dominant and is inhibited during periods characterised by dominant positivity.

The monosynaptic reflex response following stimulation of the deep fibular nerve (monosynaptic flexor response) and the multi-

synaptic response to stimulation of the sural nerve (ipsilateral flexor reflex) are facilitated during the positive dip preceding the long negative wave whereas they are scarcely influenced during the period of late negativity.

Thus excitability changes occurring during the different phases of the slow potentials have different influences on the extensor and flexor reflex activity.

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**Slow Positive and Negative Ventral Root Potentials
accompanying Extension and Flexion evoked
by a Medullary Stimulation**

By C. G. BERNHARD *and* C. R. SKOGLUND

Introduction

Slow potentials from the spinal cord have, hitherto, only been studied in reflex activity (GOTCH and HORSLEY 1891; GASSER and GRAHAM 1933; HUGHES and GASSER 1934; UMRATH 1933; UMRATH and UMRATH 1934; HUGHES, MCCOUCH and STEWART 1936; BARRON and MATTHEWS 1938; BREMER, BONNET and MOLDAVER 1942; BREMER and BONNET 1942; ECCLES 1946). Earlier papers by BERNHARD and THERMAN (1947 a) and BERNHARD (1947) have shown how flexor reflexes are followed by the development of slow, ventral root potentials with a dominantly negative phase, and extensor reflexes by similar dominantly positive potentials. These results have been discussed (BERNHARD 1947), chiefly in connection with the work of GASSER and GRAHAM.

It is apparent however from BERNHARD's work that by afferent stimulation both slow negative and slow positive potentials are involved and that it is difficult to separate these slow components by using afferent stimulation. However, SKOGLUND (1946, 1947) found that, in the cat, pure flexion or pure extension of the hind limb can be elicited by direct current stimulation of the descending motor tracts in the medulla, the response varying according to the locus and the sign of the stimulus. It seemed, therefore, of interest to study the slow potentials developed in the cord as a result of this method of stimulation. In

this paper we shall show that flexion of the hind limb is followed by the appearance of a pure negative slow ventral root potential, and extension, by a pure positive potential. It is thus possible to demonstrate that antagonistic reactions evoked from the central structures are associated with opposite electrical potentials in the ventral root.

Methods

Decerebrate cats, in which the pyramids in the medulla oblongata had been freed from the base of the skull, were used. As stimulating electrode was used the free point of an enamelled needle applied at various points in the medulla. The stimulus was monopolar and delivered from a stimulator designed to give shocks of durations varying from 0.1 to 50 msec., the discharges being taken over transformers which did not change the form of the shocks. After lumbar laminectomy, one of the roots, either L_6 , L_7 or S_1 , was cut peripherally, and the recording electrodes placed so that one was as near the cord as possible without injury and the other on the cut end of the root. The root potentials were recorded by means of a four stage amplifier, the first two stages of which were direct coupled in push-pull and the last two resistance-capacity coupled with a long time constant (1 sec.). During the experiment the animal was suspended, in moist atmosphere in a screened box, by clamps fastened to the head and the spinal processes. The legs were left free to permit movement.

Results

Composite ventral root potentials in the non-deafferented animal.

The character and localisation of the resultant limb movements depend to some extent on the position of the stimulating electrode in the medulla. Stimulation of the descending tracts often produces mixed movements, in which extension and flexion play their part, as also abduction and adduction (cf. SKOGLUND 1947).



Fig. 1. Ventral root potentials from L_7 (left side) produced by stimulation of the descending tracts in the medulla oblongata. Dorsal roots intact.
Time: 10 msec.

Figure 1 shows the record of a typical potential developed in L_7 on the left side when the stimulus evoked a mixed movement of the left hind leg. It will be seen that the initial discharge (latency 7—8 msec.) is followed by a slow polyphasic potential. Two obvious positive waves (downward displacement) with their maxima 20 and 100 msec. after the stimulus, are separated by a negative wave with its maximum at 50—60 msec. The polyphasic character of the discharge is probably due to the activation of both positive and negative processes with different time characteristics. Since this particular experiment was made on an animal in which the dorsal roots were intact, the mixed reaction may well be due partly to an in-flow of proprioceptive impulses from the contracting muscles (cf. BERNHARD 1947).

Figure 1 thus demonstrates that slow root potentials of both signs may follow stimulation of the descending tracts in the medulla.

Isolation of pure negative and pure positive ventral root potentials.

We were able to separate the negative ventral root potentials from the positive by stimulating the medulla in such a way that pure extensor or pure flexor movements could be produced. In

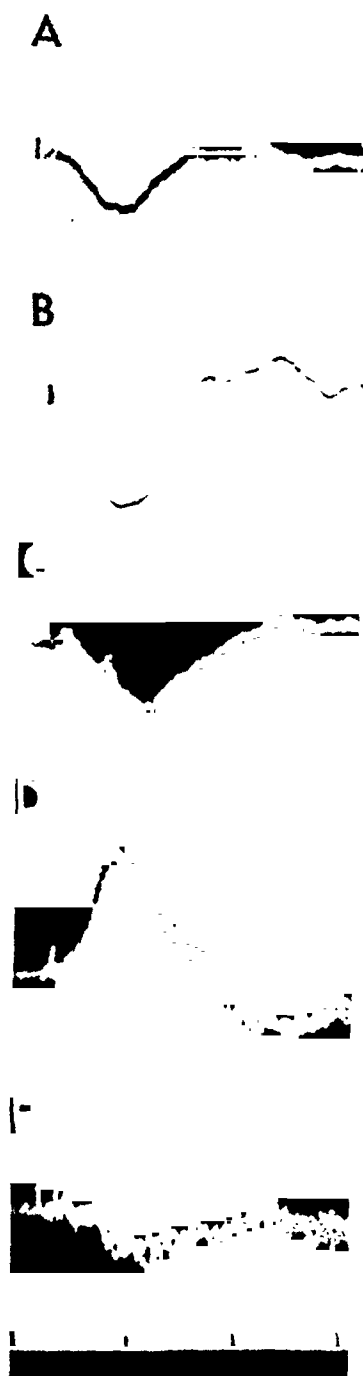


Fig. 2. Variations in the slow ventral root potentials (L_7) caused by lengthwise polarisation of the cord. Time in 100 msec. Full description in text.



Fig. 3. Ventral root potentials from L_7 (left side). Medullary stimulus. Lumbo-sacral dorsal roots cut. A. Supra-threshold stimulation causing extension of left hind leg. B. Sub-threshold stimulus. Time: 10 and 50 msec.

these experiments lumbosacral deafferentation (by dividing the sensory roots) was performed in order to exclude the effect of returning proprioceptive impulses. The application of long-duration or repetitive stimuli favoured the production of these cord potentials.

The records in Figure 2 were obtained from the ventral root as a result of a stimulus to a point in the medulla from which pure flexion of the ipsilateral hind leg could be obtained with weak stimuli. In record A the initial discharge (latency 8—9 msec.) is followed by a pure negative wave of about 60 msec. duration. Record B is taken at too low stimulus strength to evoke an initial impulse discharge; only the slow negative wave is present. Record C, from another experiment, shows a similar development of negativity in S_1 . The stimulus used to produce this record was also too weak to elicit any muscular reaction but stronger stimuli produced flexion. For this reason there is no

initial discharge and the record shows only a negative wave lasting 60—70 msec.

Potentials from the same ventral root elicited by stimuli giving extension are shown in Figure 3. In record A the initial discharge is followed by a large, slow positive wave lasting for about 100 msec., while record B, made at a lower stimulus shows the positive wave only without any initial discharge. It can thus be shown that there develops in the ventral root, 1) a pure slow wave of negativity as a result of stimulation of the descending tracts in the medulla evoking flexion; 2) a pure wave of positivity as a result of stimulation evoking extension. These high amplitude potentials can be obtained without any previous impulse discharge in the motor neurones.

Variation of the strength and form of the stimulus.

The records shown in Figure 4 demonstrate the way in which both the initial discharge and the magnitude of the slow potential increase as the stimulus strength is increased (from A—D). The great amplitude of the slow potential, in this case positive, in relation to the size of the initial discharge is especially striking.

The *duration* of stimulus is another decisive factor in determining the amplitude of the slow potential.

In Figure 5, record A was obtained with a medullary shock of 0.5 msec. duration and it will be seen that there was an initial discharge without any measurable slow potential. When, however, the same stimulus was continued for 20 msec. a large, slow positive potential appeared (record B). Exactly the same result was obtained with stimulation of the medulla causing flexion; the size of the resultant negative potential recorded from the ventral root increased with increased duration of the stimulus.

We also tried the effect of repetitive stimulation with short shocks and found that both positive and negative root potentials may be built up also by this method.

Figure 6 shows the production of a slow negative potential as the result of a series of short negative stimuli evoking flexion.

A



B



C



D

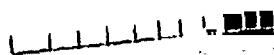
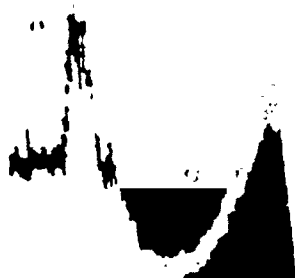


Fig. 4. Effect of increasing the strength of the medullary stimulus in the ventral root potentials. Conditions as in Figure 3. Stimulus strength increased from A—D.



Fig. 5. Effect on the ventral root potentials of increasing the duration of the medullar stimulus. Conditions as in Figure 3. A. Stimulus duration: 0.5 msec. B. Stimulus duration: 50 msec. Time: 10 msec.

We thus found that the application of either long duration or repetitive stimuli favours the building up of slow potentials in the ventral roots.

In order to obtain these slow potentials it is important that the preparation should be in good condition. It is, further difficult to evoke flexion or extension in the hind leg when the sensory roots are divided, since this operation involves a general decrease in excitability, mainly due to the elimination of the afferent in-flow. In such preparations it may sometimes be necessary to use quite strong stimuli to the medulla in order to produce any contraction in the leg muscles and the resultant movements are often mixed (cf. SKOGLUND 1947). All the experi-

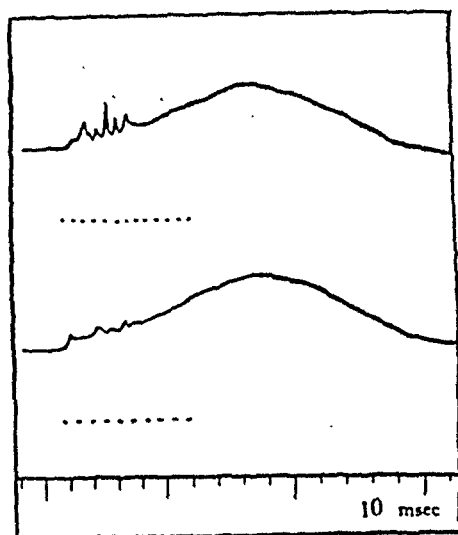


Fig. 6. Production of ventral root potentials by repetitive stimulation in the medulla. Conditions as Figure 2.



Fig. 7. Rhythmic ventral root activity following subthreshold medullary stimulation. Conditions as in Figure 3. Time: 100 msec.

ments illustrated, however, were made on animals from which pure reaction could be obtained with weak stimuli. Records from deafferented animals in good condition often show a rhythmical ventral root response to a single medullary stimulus. Such a rhythmical ventral root activity was obtained in the experiment illustrated in Figure 7, in which the stimulus strength is too low to elicit muscular contraction. When the intensity of the medullary stimulation was increased an extension was produced. In this case the series of potential changes begins with a positive wave.

Discussion

It has been shown that the slow positive and negative ventral root potentials which can be evoked by stimulation of the descending tracts in the medulla may occur without any previous discharge in these roots (Figures 2 B, C and 3 B). In consequence, they cannot be after-potentials in ventral root fibres and their local of production must, therefore, be intraspinal. Whether they are produced in the ventral horn cells and specially in their extensive dendritic network or arise in the presynaptic neurons (interneurons and terminals of descending tracts) must be left for future analysis.

GASSER and co-workers in classical investigations (GASSER and GRAHAM 1933; HUGHES and GASSER 1934), have recorded both positive and negative slow potentials from the cord following afferent stimulation. They suggested that these potentials arise from the intermediate structures. Later workers have either questioned (BARRON and MATTHEWS 1938) or shown less interest (ECCLES 1946) in the positive potentials of the ventral roots, but it has since been demonstrated (BERNHARD and THERMAN 1947 a; BERNHARD 1947) that such potentials do follow afferent stimulation. In addition, reflex extension is characterised by a dominance of positivity and reflex flexion by a dominance of negativity. It is, however, difficult when working with afferent stimulation to separate the positive and negative components of the slow cord potentials. If, on the other hand, the descending tracts of the medulla are stimulated it is often possible to obtain pure negative or pure positive potentials accompanied by flexion or extension. This experimental production of a pure positive potential in the ventral root is of particular interest, since the existence of ventral root positivity has been questioned. Our most important finding, however, is the association of root negativity with flexion and root positivity with extension.

This is not the place to go into the question of the connection between the slow potentials and the discharge of impulses from the roots, but it is possible to demonstrate definite time relations between the two phenomena. Further discussion of this

point is unlikely to be fruitful until we know more about the distribution of the potential fields on the spinal cord.

It is interesting to note that the slow rhythmic ventral root potentials (evoked by a single medullary shock, Figure 7) have the same time characteristics as the alternating periods of facilitation and inhibition of the extensor and flexor motoneurons (following a single afferent shock) studied by BERNHARD and THERMAN (1947 b).

The reason why during flexion and extension slow potentials of opposite sign could be recorded from one and the same ventral root may be that the loci of generation of the potentials are differently orientated. This would then be a valuable indication that the origin of reciprocity in antagonistic systems may be found in a definite geometric orientation of their motor neurones. On the other hand the results could also indicate different membrane characteristics of the neurones of the two systems.

Summary

1. Local monopolar stimulation of the descending tracts in the medulla evoke slow negative as well as slow positive potentials which can be recorded from the lumbosacral ventral roots. These slow potentials, affected by the proprioceptive in-flow from the muscles, can be evoked also after lumbosacral deafferentation.

2. Medullary stimulation evoking flexion is followed by a pure ventral root negativity while that evoking extension is followed by slow ventral root positivity.

3. Prolonged or repetitive stimuli, favour the production of pure slow ventral root potentials of both signs.

4. Slow potentials of high amplitude may be obtained without a previous impulse discharge.

5. The deafferented animal inclines to a special type of slow rhythmic potential activity with a frequency of 6—8 per second following medullary stimulation.

The authors are indebted to The Rockefeller Foundation and The Foundation of Therese and Johan Andersson for support of this work.

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Studies of the Potential Level in the Ventral Root under Varying Conditions

By C. G. BERNHARD, C. R. SKOGLUND *and* P. O. THERMAN

Introduction

The recording of steady potentials from various nerve structures has been described several times (see SCHLÆFER 1940), the earliest investigations on the roots of the spinal cord being made by GOTCH and HORSLEY (1891). However the fluctuations in the potential level of the ventral root have not been correlated with changes in excitability of the cord or with the activity of different functional systems.

BARRON and MATTHEWS (1938) have demonstrated that massive exteroceptive stimulation can cause the building up of long-lasting negative potentials. It has further been shown (BERNHARD and THERMAN 1947, BERNHARD 1947) that flexion and extension of the hind leg evoked by stimulation of afferent nerves are associated with the development of negative and positive potentials in the ventral roots and that the excitability in the two antagonistic systems can be correlated to these potentials of opposite sign. We have described in a preceding paper (BERNHARD and SKOGLUND 1947) how, in the cat, flexion of a hind limb evoked by stimulation of the descending tracts of the medulla is also accompanied by the appearance of a slow negative potential in the ventral roots while extension is accompanied by a similar positive potential.

While working on these ventral root potentials (recorded from a cut ventral root with one electrode near the cord and the other on the peripheral end) we noticed that the base line tended to move sometimes to the positive and sometimes to the negative side and that these displacements correspond to variations of the responses transmitted through the cord.

In this paper we have described a series of preliminary experiments in which the potential level in the cut ventral roots has been recorded under varying conditions at the same time as the excitability of the flexor and extensor systems has been studied.

Results

The method of stimulation and recording has already been described (SKOGLUND 1947 b, BERNHARD and SKOGLUND 1947). In addition we studied changes in the potential level in the ventral root by means of a Cambridge spot galvanometer coupled to the first two direct-coupled stages of the amplifier.

Preliminary experiments demonstrated that there may be extreme fluctuations in the potential level recorded from a cut ventral root (L_7) with one electrode near the cord and the other on the peripheral end.

We attempted to produce regular changes in the potential level recorded from the ventral root by changing the afferent in-flow. This was accomplished simply by changing the position of one of the hind legs. The typical result of such an experiment is shown in Fig. 1. The cat was decerebrate and the lumbo-sacral sensory roots on the left side had been cut so that afferent impulses could be transmitted from the right but not from the left hind leg. The potential level was recorded from L_7 on the left side while the right hind leg was moved. The beginning of each curve in Fig. 1 indicates the potential level recorded before each observation and it can be seen that flexion (F) of the right leg caused an upward displacement of the curve while stretching (E) caused a downward displacement. It will be seen that the change in the potential level which accompanied a change in the position from extreme flexion to extreme extension or vice versa

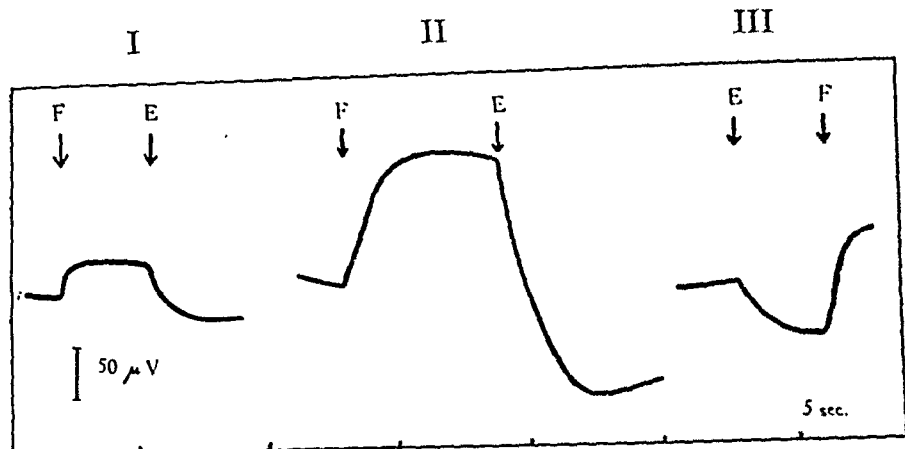


Fig. 1. Changes in potential level recorded from the left ventral root (L_7) by changing the afferent in-flow from the right leg. At F the position of the right hind leg was changed to extreme flexion and at E to extreme extension. One electrode was near the cord and the other on the cut end of the root. Upward displacement corresponds to an increased negativity under the proximal electrode and vice versa.

was variable; in curve I the difference is about 50 microvolts, in curve II about 400, while in curve III where the leg was first stretched and then bent there was a final increase in negativity of about 100 microvolts. Comparable displacements in the level of the steady potentials were also obtained with movements of the ipsilateral leg in animals with intact afferents.

It was also found that the extensor twitches of the left hind leg evoked by stimulation of the descending tracts in the medulla varied in size and that such variations in the responses were related to the fluctuations in the steady potential of the ventral root. For instance, an upward displacement of the base-line (increase in negativity under the proximal electrode) was correlated with increase of the extensor responses and vice versa. The flexor responses also varied with changes in the potential level, so that a decreased ventral root negativity was associated with increased flexion.

These experiments, therefore, show that muscle responses evoked by medullary stimulation (flexion or extension) vary in strength with variations in the level of the steady potential of the ventral root.

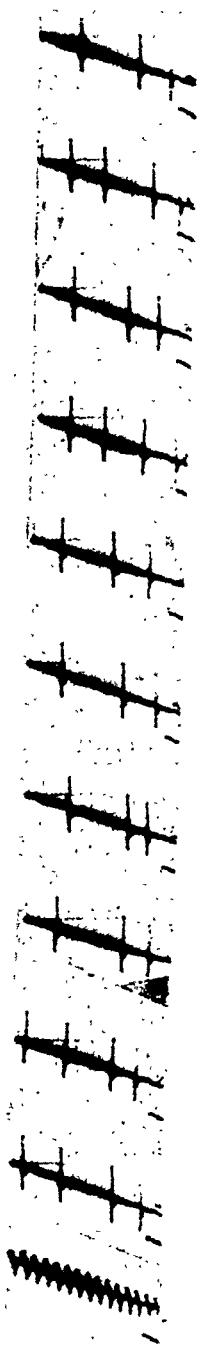


Fig. 2. Single unit potentials from the biceps muscle of the left foreleg during slight tension. Each record shows spike distribution after a stimulus applied to the right sciatic nerve. Time in 10 msec.

Table I.

	Exp.nr.	Influence on extension and accompanying positive ventral root potential decrease (—) increase (+)	Influence on flexion and accompanying negative ventral root potential decrease (—) increase (+)	Influence on ventral root potential level towards positivity (+) or towards negativity (—)
<i>Acetylcholine</i>	12	—	+	
	15	—	+	
	15	—		
	16		+	—
	16		+	
	19		+	—
	19		+	
	19A			— 0.5 mV
	19A			— 1.5 mV
	20			— 0.5 mV
	21			— 0.5 mV
<i>Adrenaline</i>	13	+		
	15			+
	15	+		+
	15			+
	14			—
	16			+
	16			+
	16A			+
	16A			+
	16A			+ 0.2 mV
	19A	+	—	+ 2.5 mV
	19A	+	—	+ 0.8 mV
	20			+ 1.5 mV
	20			+
	21			+ 1.0 mV
	21			+ 1.0 mV

Since this natural potential field in the cord seems to determine the sensitivity of the different functional systems, the next step was to investigate the effect of an artificially applied electrical field. We investigated the effect of lengthwise polarisation of the cord on the slow positive and negative ventral root potentials following extension and flexion evoked by stimulation of the medulla (see BERNHARD and SKOGLUND 1947). The polarising

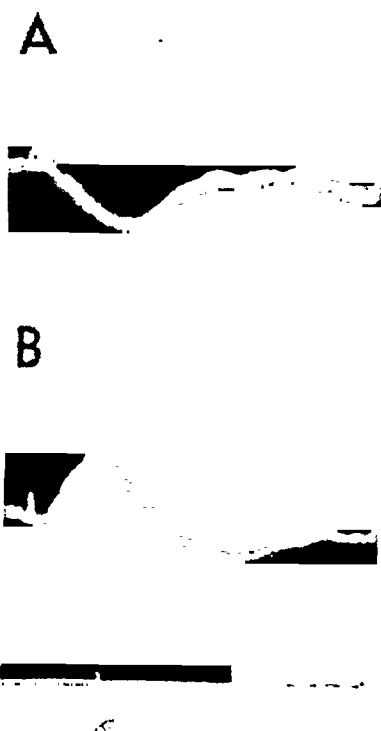


Fig. 3. Influence of acetylcholine on the slow positive ventral root potential. A. Before the application of Ach. B. Some seconds after the application of Ach. Time in 100 msec. Full description in text.

electrodes were placed about 5 cm apart on the dorsum of cord one cranially and one caudally of the root in question.

The effect of polarisation is shown in Fig. 2. The positive slow potential of record A was evoked by medullary stimulation and was accompanied by extension of the left hind leg when the stimulus strength was increased. In record B this slow positive wave is increased in amplitude by polarisation of the cord with the cranial electrode positive. Record C shows the return of the positive wave to its initial size at cessation of polarisation. When the cord was polarised in the opposite direction the first effect was to decrease the size of the slow positive potential. As the strength of polarisation was increased the effect on the slow potential was so great that finally its sign changed and it became negative (record D). At the same time the extensor responses

were replaced by flexor responses. When polarisation was stopped the slow potential became positive once more (record E). An analogous effect of polarisation was observed on flexor responses and associated negative root potential evoked by medullary stimulation. As one would expect, the direction of polarisation favourable to extension and a positive root potential was unfavourable to flexion and a negative potential and vice versa.

We then attempted to produce similar changes in the functional state of the spinal cord by the application of acetylcholine and adrenaline. Acetylcholine (1 in 10,000) and adrenaline (1 in 10,000) were dropped on the exposed lumbar region of the cord at the level from which the records were taken. In every case application of acetylcholine resulted in an increase in the steady negativity of the ventral root (500 to 1500 microvolts, see Table 1). At the same time flexion of the hind leg evoked from the medulla increased in strength and the allied slow negative potential from the ventral root increased in size, while the extensor responses and the slow positive potential were decreased. It was even possible to convert extension into flexion and a positive root potential into a negative one by application of acetylcholine (Fig. 3).

Adrenaline, on the other hand, decreased the steady negativity of the ventral root in all experiments except one, and this resulted in an increase of extensor activity in the leg muscles and a decrease of flexor activity. At the same time extensor positivity evoked by medullary stimulation was increased and flexor negativity decreased.

Discussion

In the first series of experiments described in this paper we have used a physiological method of producing changes in the potential level by altering the tension in the muscles of the legs. It is obvious that the method of moving the whole leg involves very complex changes in the proprioceptive in-flow. However, one conclusion can be drawn from the results of the present investigation. Since the size and character of the responses evoked by medullary stimulation bears a certain relationship to the changes in the potential level this may be taken as a measure

of the state of intraspinal excitability. In other words, our results suggest that electrical fields in the spinal structure exert a reciprocal influence on antagonistic systems.

The experiments in which the cord was polarised shows that functional changes analogous to those described above can be produced by artificially altering the normal state of polarisation in the cord. The opposite influence by polarisation on the excitability of the two systems is in accordance with results from experiments in which the activation of the systems by direct current stimulation was studied (SKOGLUND 1946, 1947 a).

The potential level may be affected also by the application of different substances. Acetylcholine and adrenaline seem to have opposite effects on the potential level recorded from the ventral root, as well as on the excitability of the antagonistic systems. In preliminary experiments BERNHARD and THERMAN (1947) also observed that acetylcholine and adrenaline had similar influence on the slow potentials recorded from the spinal roots. It is too early to draw a comparison between our results and the observations made on the effects of acetylcholine and adrenaline on spinal reflexes evoked by afferent stimulation. Both similar and opposite effects of these two substances on extensor and flexor reflexes have been demonstrated (SCHWEITZER and WRIGHT 1937, BÜLBRING and BURN 1941).

The fact that the influence on the excitability of the potentials evoked by changing the afferent in-flow is different to that produced by the application of drugs does not necessarily mean that the potentials are of different nature. In order to more clearly interpret our results it is necessary to have a wider knowledge of the geometrical orientation and the excitability properties of the neurones in the different antagonistic systems.

Summary

1. The potential level from the proximal part of the cut ventral roots (L_7 and S_1) has been recorded under varying conditions, at the same time as the excitability in the flexor and extensor systems has been studied.

2. This potential level has been shown to vary with changes in the proprioceptive in-flow and with local application of acetylcholine and adrenaline.

3. The displacement of the potential level, evoked by these methods, is always accompanied by changes in the excitability of the antagonistic flexor and extensor systems so that displacement in one direction corresponds to increased excitability in the flexor system and decreased excitability in the extensor system, while a displacement in the opposite direction corresponds to an opposite effect. Artificial polarisation of the cord in lengthwise direction produces analogous results.

The authors are indebted to The Rockefeller Foundation and The Foundation of Therese and Johan Andersson for support of this work.

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Responses of Different Types of Nerve Fibres to Ascending and Descending Currents

By C. v. EULER and C. R. SKOGLUND

Introduction

The phenomenon that antagonistic systems in the spinal cord of the cat are activated, when direct currents of opposite direction are applied, may be due to differences in anatomical orientation of the elements in relation to current flow or to different inherent properties of the excitable structures. (SKOGLUND 1946, 1947.)

Although it is quite clear that further experimentation on the spinal cord is necessary in order to obtain more definite information of the factors involved, it is nevertheless tempting to investigate whether or not model experiments on peripheral nerves can throw some light on the mechanism of the central excitation processes. A systematic study of the responses of different nerves to ascending and descending stimuli was made in order to see if phenomena corresponding to those in the spinal cord could be evoked in peripheral nerves.

As is shown in the present paper, it was actually found that fibre groups can be separately activated by ascending and descending currents. An analysis of the different factors causing the selective effect revealed *e. g.* that while some fibres were most effectively stimulated by descending currents, other fibres had a lower threshold for ascending currents. Some results are also presented from a comparative analysis of the loci of generation of impulses in differently reacting fibres.

Methods

The nerves were excised from decerebrate cats and placed in Krebs solution held at a constant pH of about 7.35 by bubbling a mixture of oxygen (95 %) and carbon dioxide (5 %) through the solution. [Krebs solution: NaCl (0.154 *M*) 100, KCl (0.154 *M*) 4, CaCl₂ (0.11 *M*) 3, KH₂PO₄ (0.154 *M*) 1, MgSO₄ · 7H₂O (0.154 *M*) 1, NaHCO₃ (0.154 *M*) 21 volume parts; 1 g. glucose per liter solution.] During the recording the nerves were kept in a moist nerve chamber having an atmosphere of the above composition and a constant temperature of 35° C. The stimulating electrodes were of chlorided silver wires of 0.5 mm. diameter. The various arrangements of the electrodes in different experiments are shown in the figures. In our standard arrangement one of the stimulating electrodes is placed on the crushed end of the nerve and the other at a distance of 10 mm. The stimulus form was recorded on one beam of the cathode ray tube via a direct-coupled amplifier. Due to different amplification the deviation of this beam is not proportional to the stimulus strength in all experiments. The absolute value of the stimulating current was read on an ammeter in series with the preparation. The second beam of the tube was connected with a condenser-coupled amplifier for recording the nerve impulses.

Results

A fibre responses. Both make and break responses have been recorded in our experiments but only make responses will be analysed in the present paper. The effects of stimulation described below always refer to make responses, if not otherwise stated.

The cat's A fibres, investigated *in situ*, have a lower threshold for descending than for ascending currents, (see *e. g.* SKOGLUND 1945) in accordance with Pflüger's (1859) classical results on frog nerves. In the excised nerves, studied under the conditions described above, A fibres exhibited the same threshold difference, as demonstrated in Fig. 1. Record *a* shows the characteristic rhythmical responses of the phrenic nerve to a descending current of a given strength, while in *b*, after reversal of the current, no

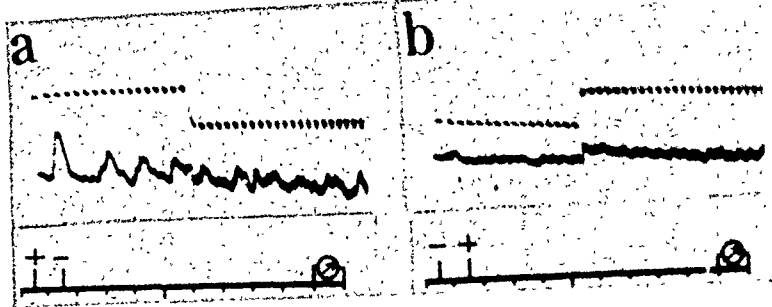


Fig. 1. Responses of the phrenic nerve to stimulation with rectangular currents of (a) descending and (b) ascending direction. Stimulus form and time marking on the upper beam. Time intervals 2 msec. The electrode arrangement is shown by the diagram below each record. The nerve length in the diagrams is divided in centimeters.

response is observed. A considerable increase in stimulus strength was necessary in order to produce a similar response as in *a*.

Typical responses of A fibres to currents of opposite direction are further illustrated in Fig. 2, obtained from an experiment on the cervical part of the vagus nerve. In records *a*—*d* the stimulation was applied in descending direction, in *e*—*h* in ascending direction. Record *a* shows a sub-maximal α response. The maximal α potential in *b* is followed by small repetitive discharges superposed on slower waves. The repetitive responses are more marked in *c* and *d* where the stimulus strength is sufficiently high to produce responses from the C fibres as well (cf. below). Record *e*, obtained with the same strength as in *a*, shows that the A response to the ascending current is smaller than to the descending current. A maximal A wave could be obtained when the stimulus strength was increased (*f*). Further increase, however, caused first a reduction (*g*) and finally a complete abolition of the A potential (*h*). This characteristic inhibition is very pronounced when ascending current pulses of more than a few msec. are applied.

The typical behaviour of the A fibres, from an experiment on the genito-femoral nerve, is again demonstrated in Fig. 3. Records *a*—*d* show that the initial spike potential is followed by repetitive discharges during the plateau phase of the descending currents. A striking contrast to this behaviour is the complete

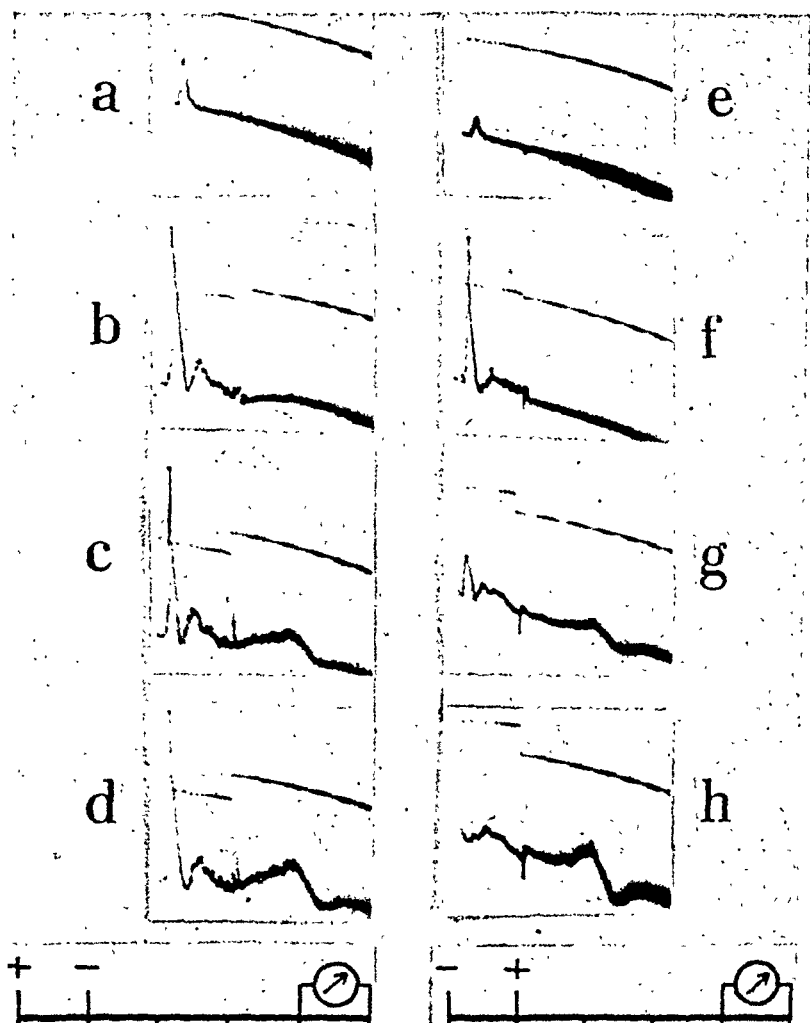


Fig. 2. The cervical part of the vagus. Stimulation with (a—d) descending currents and (e—h) ascending currents. Stimulus duration 22 msec. Time intervals 2 and 20 msec. Sweep velocity falls off logarithmically. The C waves (conduction velocity about 0.7 m./sec.) are make responses.

absence of any type of A response to the ascending currents in records e—h. The inhibition of the A fibres in this nerve was so dominant that even at very low strengths of ascending currents no responses were elicited at make; excitation occurred first at break of the stimulation (to the right in the records).

A lower threshold for descending than ascending currents was typical for all A fibres investigated, as seen in Table I (p. 10),

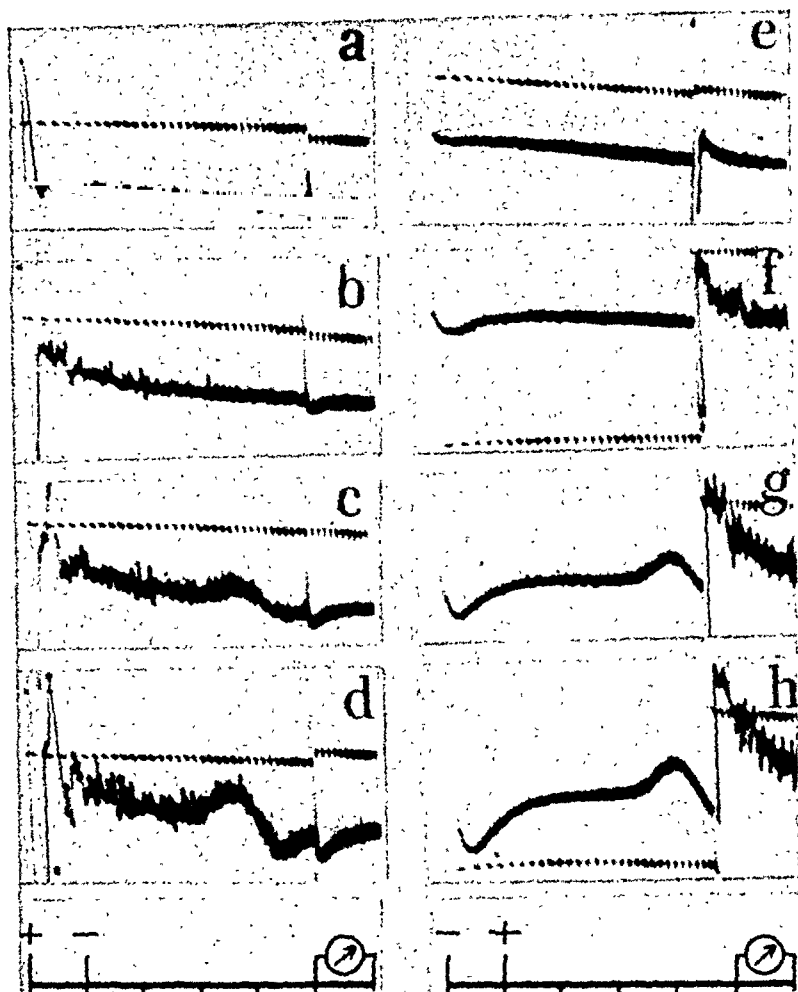


Fig. 3. The femoral branch of the genito-femoral nerve. Stimulation with (a—d) descending and (e—h) ascending currents. There is no proportionality between the strength of the stimulus and the deflection of the beam recording the stimulus form in the different records. Stimulus duration about 60 msec. Time intervals 2 msec. Full description in text.

where the different observations are collected. The relation between the thresholds can be influenced by many factors, *e. g.* the electrode positions or the chemical milieu (cf. SKOGLUND 1945). It is obvious that the uniform results in the present series of experiments are due to the constant conditions in the nerve box. All data collected in the table are from experiments in which the standard electrode arrangement was used (see Methods). Special

experiments in which systematic changes of the electrode positions were made showed, however, that the threshold relation remains the same even when the interelectrode distance is increased up to several centimeters or both electrodes are moved to an intact region of the nerve.

Selective activation of A and C fibres. The records in Figs. 2 and 3 are not only selected in order to illustrate the well-established behaviour of the A fibres but also to show the selective activation of the C fibres by ascending currents. We will first draw attention to the striking differences between records 2 *d* and *h*. While both A and C fibres are activated by the descending current in *d*, only the C potential appears in *h* after reversal of the current. The separate activation of C fibres at make, without any responses from other fibre groups, is clearly demonstrated in Figure 3 *g* and *h*, although the stimulus duration is such that the pronounced responses at break appear just at the end of the descending phase of the potential.

A discussion of the factors causing selective activation is appropriate at this point. It appears from Fig. 2 *g* that the depression of the A fibres is first observed when the stimulus strength corresponds approximately to the threshold of the C fibres. Fig. 2 *h* shows that further increase in stimulus strength causes depression of the A but not of the C potential.

The descending currents activate A fibres and the ascending only C fibres, when the stimulus strengths are adjusted to suitable values (cf. Fig. 2 *b* and *h*; 3 *b* and *g*). In the experiment in Fig. 3 selective activation of the two fibre groups by simply reversing the current direction would have been possible if the thresholds of the C fibres had been lower for ascending than descending currents. In the experiment in Fig. 2 the threshold of the C fibres was in fact lower for ascending than for descending currents (120 μ A and 165 μ A respectively). However, this fact was of no importance for the selectivation in this experiment because below 165 μ A the A fibres were not completely depressed. It appears from this brief discussion that the threshold values for both excitation and inhibition in the two fibre systems are important

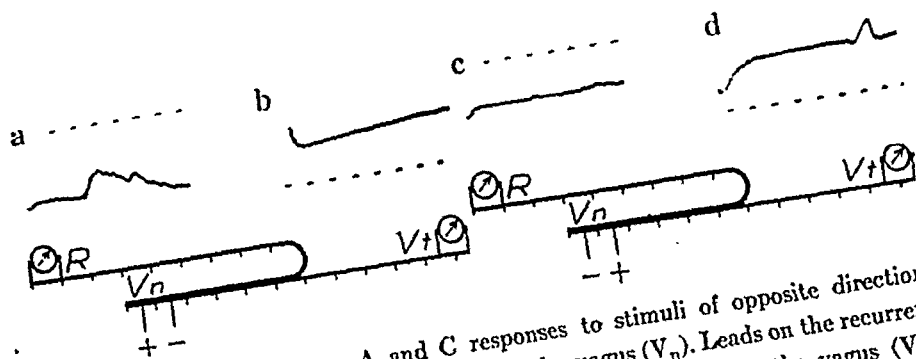


Fig. 4. Comparison between A and C responses to stimuli of opposite direction. Stimulating electrodes on the cervical part of the vagus (R). Leads on the recurrent nerve (R): records a and c. Leads on the lower thoracic part of the vagus (V_n): records b and d. Stimulus duration 20 msec. Time intervals in a and c 1 msec.; in b and d 20 msec.

factors in the selective activation, illustrated in Figs. 2 and 3. Under the heading Selective activation of B and C fibres another example will be given.

C fibre responses. A systematic study of different nerves was made in order to determine whether the observation of an opposite relationship between the thresholds of the C fibres as compared to A fibres (in the experiment shown in Fig. 2) was significant.

Six vagus nerves were examined and in five of these the C fibres had a definitely lower threshold for ascending than for descending currents, while one showed the reversed reaction. The most common type of response is illustrated in Fig. 4, where at the same time a comparison is made with the A fibres by placing the stimulating electrodes on the vagus in the neck and recording the A and C waves from the recurrent nerve respectively the vagus nerve in the lower thoracic region. According to CHASE and RANSON (1914) and HEINBECKER and O'LEARY (1933) the vagus in this region contains almost exclusively C fibres. Record a shows the A response (note the high sweep velocity) to a descending current of given strength; record c demonstrates the absence of response after reversal of the current. It can be seen from records b and d that the C fibres behave diametrically opposite to A fibres: d shows the maximal C response to the ascending current, and b shows the lack of response to a descending current of the same strength.

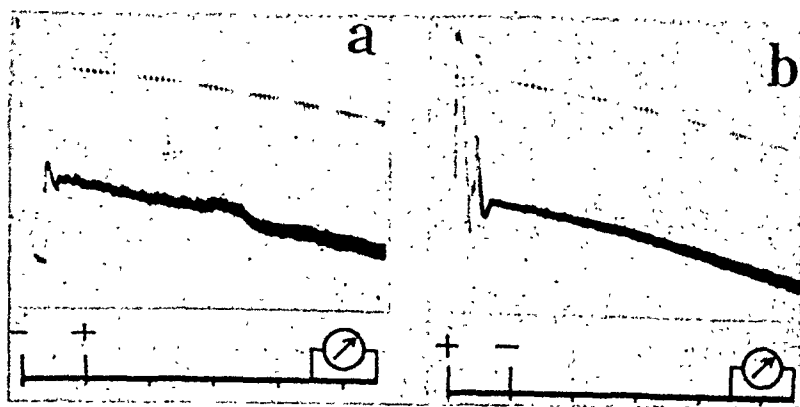


Fig. 5. The saphenous nerve. Stimulation in *a* and *b* with currents of the same strength but of opposite direction. Conduction velocity of the C wave in record *a* is about 0.75 m./sec. Time intervals 2. and 20 msec. Full description in text.

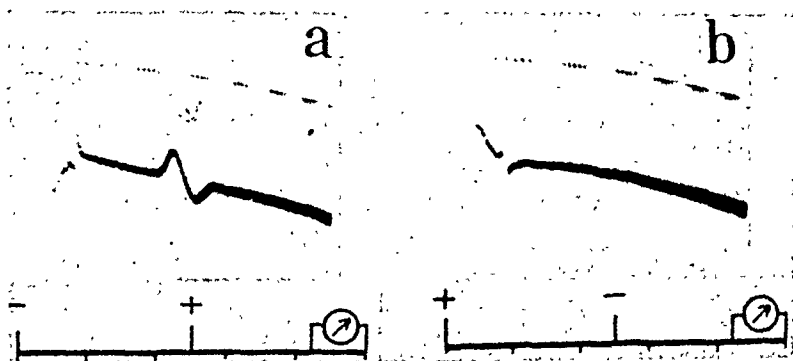


Fig. 6. Responses of one of the accelerans branches from the right stellate ganglion to the heart. The same stimulus strength in both records. Time intervals 2 and 20 msec.

The C fibres in three out of four genito-femoral nerves were stimulated at a definitely lower threshold by ascending than by descending currents. The C fibres in the saphenous nerve showed the same threshold difference as illustrated in Fig. 5. In record *a* an obvious C fibre response is obtained by an ascending stimulus of 120 μ A while in *b*, after reversal of the stimulus, a current strength of 160 μ A caused no excitation of the C fibres.

The C fibres were further investigated in the accelerans and the typical result is seen in Fig. 6. A current which gives a

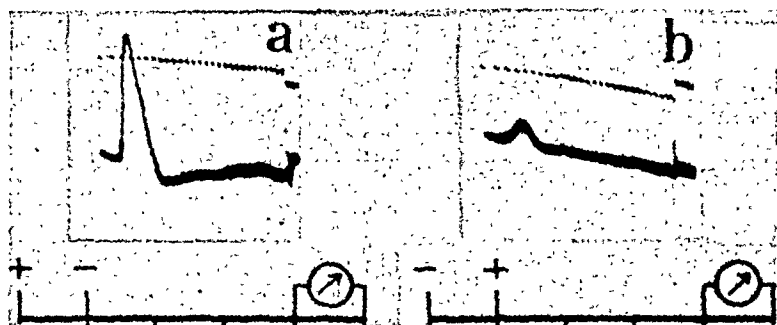


Fig. 7. Responses of the cervical sympathetic trunk to stimulation with (a) descending and (b) ascending currents of the same strength. Time intervals 2 and 20 msec.

maximal C wave when applied in ascending direction (a) is without excitatory effect when reversed (b).

The C potential in the cervical sympathetic was usually of small voltage (cf. BISHOP and HEINBECKER 1932). In the two cases where it was possible to determine the threshold relation, it was found to be the same as that of the accelerantes.

All the hypogastric nerves investigated were most effectively stimulated by ascending currents.

It can thus be concluded (cf. Table I) that the C fibres are, as a rule, stimulated at a lower threshold by ascending than by descending currents under the conditions for these experiments.

B fibre responses. The study of the B fibres in the vagus was rendered difficult by the repetitive A fibre activity and therefore no threshold determinations of this fibre type were made. The B fibres in the cervical sympathetic trunk were tested in several different nerves and in all of them the B fibres showed the typical behaviour seen in Fig. 7, *i.e.* a greater response to a descending than to an ascending current of the same strength. The B fibres in the hypogastric nerves also showed the same relation between the thresholds. As appears from Table I, the analysis of the B fibres was not very extensive. However, all the nerves investigated showed the same behaviour as the A fibres. This result is in accordance with GRUNDFEST'S (1939) statement that in many respects B fibres behave more as thin fibres of the A group than as C fibres.

Table I.

Nerve	Fibre class		Nerve	Fibre class	
	A	C		B	C
Sciatic	—		Cerv. sympathetic	—	
Phrenic	—		»		+
Saphenous	—	+	»	—	+
Genito-femoral	—	+	»	—	
»	—	—	»	—	
»	—	+	»	—	
»	—	+	Hypogastric	—	+
Vagus	—		»		+
»	—	+	»	—	+
»	—	+	»	—	+
»		+	»		+
»	—	+	Accelerantes		+
»	—		»		+
»	—	—			
»	—	+			

— = lowest threshold for descending currents (*i. e.* cathode towards the leads).

+ = lowest threshold for ascending currents (*i. e.* anode towards the leads).

Selective activation of B and C fibres. A comparison of B and C responses to stimuli of opposite direction was made in experiments on the hypogastric nerve. These nerves showed a dominating C wave and a small B potential (cf. GRUNDFEST and GASSER 1938). Fig. 8*a* shows that a descending current of a strength corresponding to a maximal B wave is subthreshold for C fibres. The threshold of the C fibres is attained first when the current strength is increased (*b*). Record *c* shows maximal B and C waves.

When the current is reversed, the threshold of the B fibres is raised and that of the C fibres lowered. This makes a selective activation of either B and C possible by simply reversing the current direction, as shown in Fig. 9. In record *a* the strength of the descending current is such as to produce a maximal B wave but no C potential. When the polarity of the stimulus is reversed without changing the current strength, the picture is opposite as seen in record *b*: a maximal C wave but no B potential.

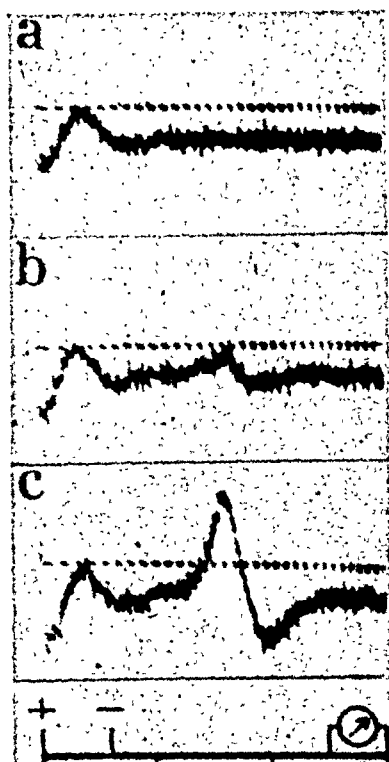


Fig. 8. Responses of the hypogastric nerve to descending stimuli of successively increasing strength. Record *a*, maximal B wave only (conduction velocity about 5 m./sec.); *b*, maximal B wave and threshold C response (conduction velocity about 0.75 m./sec.), *c*, maximal B and C waves.

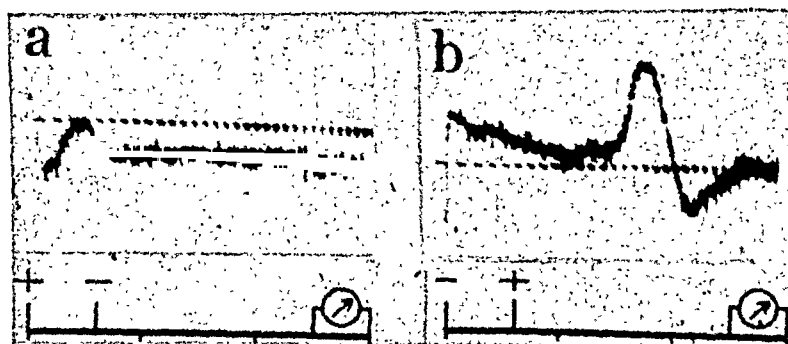


Fig. 9. Hypogastric nerve. *a*, B response to descending current, *b*, separate C response to ascending current of the same strength. Stimulus duration 4 msec. Time intervals 2 msec. See text.

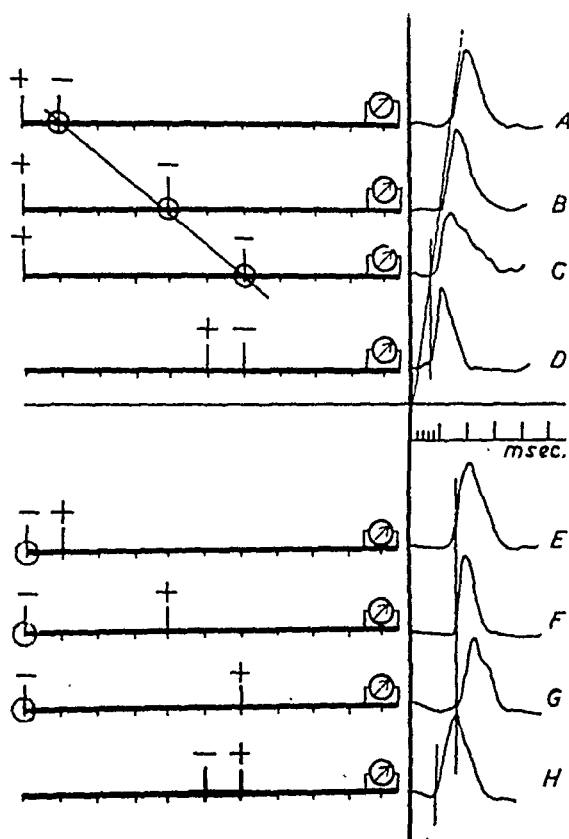


Fig. 10. Phrenic nerve. Various arrangements of the stimulating electrodes are shown in the diagrams to the left; corresponding A fibre potentials to the right. Open circles: locus of excitation. See text.

Loci of excitation in A and C fibres. It is generally accepted that in A fibres, which have their lowest threshold of excitation when the cathode is towards the leads, the impulses are generated at the region of the nerve in contact with the cathode. It is of interest to see whether the locus of excitation is the same or not in C fibres, which have the lowest threshold of stimulation when the anode is towards the leads. Some results from a comparative analysis of A and C fibres are presented. In these preliminary experiments the method used to determine the locus of excitation was to change the positions of the stimulating electrodes; a rather primitive method which nevertheless throws some light on the question.

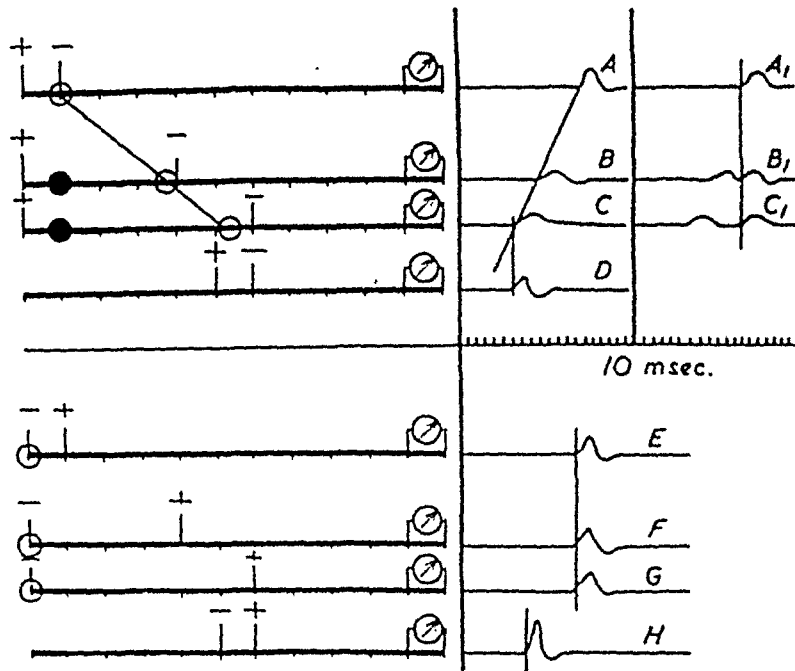


Fig. 11. Vagus nerve. Electrode positions to the left; corresponding C fibre potentials to the right. Open and filled circles show the different loci of excitation. See text.

A typical experiment on the A fibres (in the phrenic nerve) is first illustrated, Fig. 10. It appears from a comparison between the diagrams *A*, *B* and *C* that, when the cathode is moved along the nerve, the latencies of the A potentials become successively reduced in proportion to the shortening of the distance between the cathode and the leads. Assuming that the locus of excitation, marked by an open circle in record *A*, is at the cathode, by calculating the loci of excitation in records *B* and *C* from the latencies of the respective potentials, the origin of impulses is then found to be exactly under the negative electrode in both *B* and *C*. The calculations are made on the presumption that the conduction speed is not influenced by the altered electrotonic conditions; nor can the possible error be very great since the electrode arrangement in record *D* gives the same spike latency as in *C*.

Records *E*—*G* show the results when the anode is moved. The latency now remains constant showing that the impulses are still originated at the cathode. Only when the negative electrode

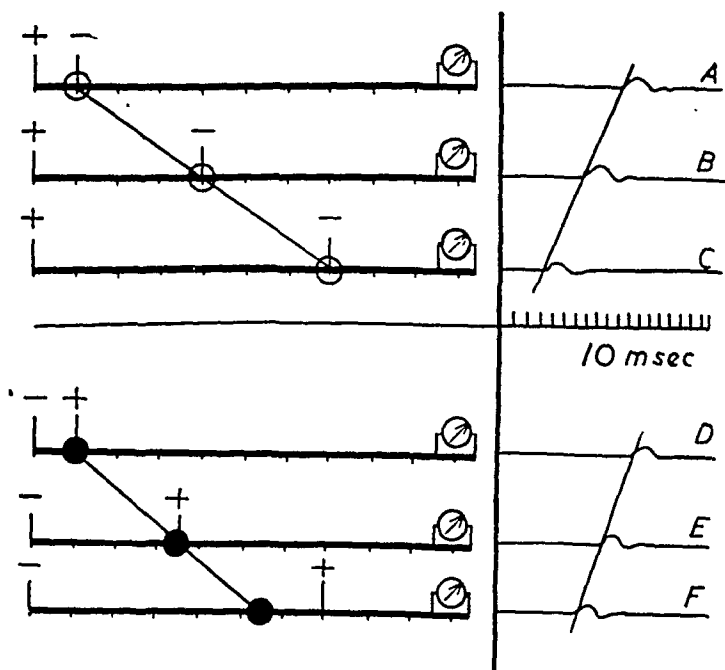


Fig. 12. Vagus nerve. Experimental conditions as in Fig. 11. See text.

is moved a change in latency can be observed (*H*). The results obtained in experiments from A fibres by this method are thus quite clear.

Fig. 11 shows the results from a similar experiment on C fibres having the typical threshold relation. Records *A—D* and *E—H* show that, when descending and ascending stimuli are applied, the loci of excitation are exactly the same as in experiments on A fibres. We noticed, however, one difference; when the stimulus strength of the descending current was increased above the maximal C potential (records *B₁* and *C₁*), double waves appeared. The origins of these new waves, calculated from their latencies, were found to be at the points marked by filled circles. The simplest interpretation of this result is that excitation took place at two different points on the nerve simultaneously, one near the cathode, the other near the anode. It was observed in all experiments on the C fibres that excitation can take place both at the cathode and at the anode. This is most clearly shown in the experiment in Fig. 12. Records *A*, *B* and *C* show that the locus of excitation is at the cathode, when descending stimuli are

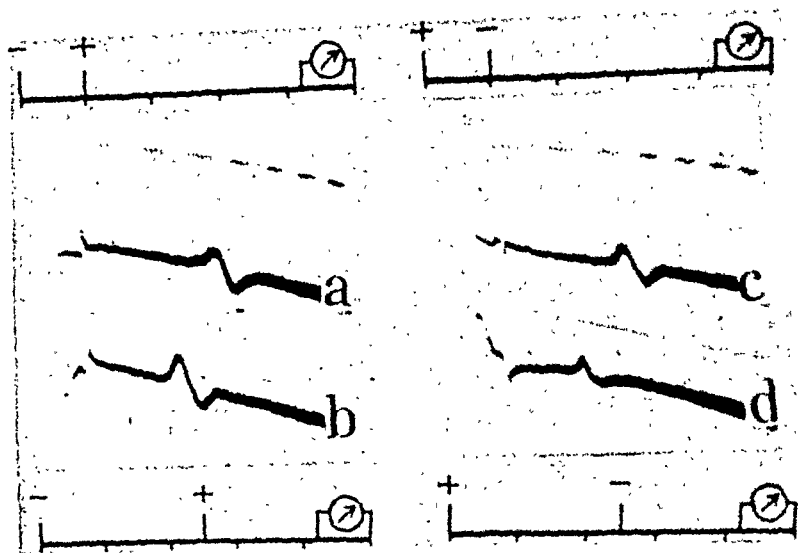


Fig. 13. The accelerantes. The upper diagrams refer to records *a* and *c*; the lower diagrams to *b* and *d*. Time intervals 20 msec. See text.

applied. If the current is reversed and the anode is moved, however, the latencies do not remain constant as in the previous experiments (Figs. 10 and 11) but become successively shorter. Whether the excitation in *D* takes place at the cathode or at the anode cannot be determined, but if the loci of excitation in *E* and *F* are calculated as before they are found to be situated at the points marked by the filled circles, *i.e.* under the anode in *E* and nearer the anode than the cathode in *F*.

The same result is also illustrated by some records from an experiment on the accelerantes, Fig. 13. The records show that the latencies become shortened both when the cathode and when the anode is moved towards the leads.

In all these experiments the thresholds of the C fibres have been lower for ascending than for descending currents, irrespective of the electrode positions.

Discussion

Our investigations have given a positive answer to the question, raised in the introduction, whether or not in peripheral nerves different fibre systems can be activated by stimuli of opposite

direction. The results obtained, namely, that one fibre group is activated by a current of a given direction and inhibited by a current of opposite direction concurrently activating a different fibre system, show an interesting analogy to the similar phenomena of excitation and inhibition in the spinal cord.

In the course of the investigation our attention was drawn to the relation between thresholds for ascending and descending currents, a problem which, with a few exceptions, has not been studied with the help of modern methods. ROSENBLUETH (1941) found in experiments on cat nerves *in situ* that the thresholds for descending and ascending currents at make varied with different electrode positions, so that no fixed relationship could be determined. SKOGLUND (1945) found that at a given electrode position constant threshold values were obtained in cat nerves, while in excised frog nerves the thresholds were variable.

In the present experiments on excised cat nerves it was surprising to find threshold values of such a constancy, that a differentiation between fibres was possible. It must be emphasized that the typical differences between the respective fibre classes are based only upon investigations of the nerves specified in Table I and therefore the results should not be generalised. This applies particularly to the B fibre group which has been studied only in a few different nerves. A further analysis may also reveal threshold differences between fibres in one and the same group. Certain differences between the responses of autonomic and somatic C fibres to direct current stimulation have already been demonstrated (C. v. EULER 1947).

We have deliberately used the terms descending and ascending currents instead of cathodal and anodal stimulation for the following reasons. First, as appears from the last section, special investigations are necessary in order to determine whether excitation takes place in the region of the nerve at the cathode or at the anode. Secondly, even if the excitation is localised to the neighbourhood of the anode, impulses may in fact originate from a spurious cathode. Membrane potentials have been demonstrated at the anode which are different from those at the cathode (in *Sepia* nerves by ARVANITAKI and CARDOT 1939, ARVANITAKI 1943 and in frog nerves by LORENTE DE NÓ 1947), but even

here it has been demonstrated that the impulses appear on the negative phase of the fluctuations in the membrane potentials. According to LORENTE DE NÓ excitation in frog fibres by the closure of the anodal currents occurs only when the nerve is in a rhythmical state and when weak stimuli are used. Although these conditions do not exist in our experiments on C fibres, excitation does occur in the region of the anode. No studies of the membrane potentials have been made in our present investigations. It is interesting, however, to note that the latency of the responses evoked by ascending currents is always longer than that by descending currents, which might indicate that the membrane processes in the two cases are different also in cat C fibres.

The different thresholds for ascending and descending currents are not easy to explain on the basis of our present knowledge (cf. SCHAEFER 1940). The contrary behaviour of A and C fibres can be fully understood only when the membrane properties of the C fibres are as well-established as those of the A fibres.

Summary

The responses of different types of nerve fibres to stimulation with direct currents of descending and ascending direction have been studied in excised cat nerves. Make responses only have been analysed.

Under the given experimental conditions, fibres of different groups behave as follows:

A fibres (in the sciatic, phrenic, vagus, genito-femoral and saphenous nerves) are stimulated at a lower strength by descending than by ascending currents.

B fibres (in the cervical sympathetic and the hypogastric nerve) are most effectively stimulated by descending currents.

C fibres (in the vagus, genito-femoral, saphenous, hypogastric and accelerantes nerves) have a lower threshold for ascending than for descending currents.

C fibres can be separately activated in mixed nerves by stimulation with direct current of ascending direction.

Preliminary experiments have been made in order to analyse the excitation processes in nerves reacting differently. The experiments show that while in A fibres the excitation at make always takes place near the cathode, the make excitation in C fibres may also occur near the anode.

Special attention is directed to the parallellism between the selective activation of different fibre systems in peripheral nerves and of different systems in the spinal cord evoked by reversing the polarity of the stimulus in both cases.

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**On the Functional Organisation
of the Motoneurons in the Lumbo-Sacral Segments
of the Spinal Cord**

By K. E. ÅSTRÖM

Introduction

It has long been known that the cell-bodies of the motoneurons are arranged in groups (columns) of characteristic shape within the grey matter of the spinal cord (VAN GEHUCHTEN and DE NEEF 1900, ROMANES 1941, ELLIOTT 1942 and others). This arrangement may be of functional significance, but despite several attempts to obtain a correlation between individual muscles and different cell-columns (MARINESCO 1904 and others), no generally accepted interpretation has been found (see ELLIOTT 1942).

The investigations referred to, only deal with the cell-bodies and not with dendrites of the motoneurons nor with premotor elements (interneurons, afferent collaterals etc.).

The fundamental analysis of the central synaptic transmission made by LORENTE DE NO (see *e. g.* 1938, 1939 and 1947) has brought to light the importance of correlating anatomical and physiological data in the study of the synaptic transmission to motoneurons. It is apparent that an analysis of the functional orientation of the motoneurons in the spinal cord requires a study of all structures, the cell-body and the axon being only the last functional stage. LORENTE DE NÓ (1938) also has emphasized our incomplete knowledge of the functional organisation of the

dendrites in the spinal cord (cf. BERNHARD and REXED 1945, WOOLSEY 1947).

The question concerning the orientation of the motoneurons with antagonistic functions is of great interest in connection with recent observations on the antagonistic effects of artificial stimulation of the spinal cord with currents of opposite direction (SKOGLUND 1946, 1947) and also with observations on the slow ventral root potentials of opposite sign following reflex activity in antagonistic systems (BERNHARD and THERMAN 1947, BERNHARD 1947). These phenomena may indicate a difference in the geometrical orientation of antagonistic motoneurons (cf. LOEB 1918) and their dendrites.

The explorative experiments to be described in this preliminary report deal with a functional analysis of the dendritic ramifications of motoneurons at the boundary between the L_7 and S_1 segments of the spinal cord. The experiments are based on a correlation of electrophysiological and histological data.

Methods

21 experiments have been performed on 10 cats under dial anaesthesia (0.5 cc per kg.). The dorsal roots were cut bilaterally from L_5 to S_2 . A fine needle electrode insulated except for the point (diameter not exceeding 0.03 mm) was used for microstimulation of the spinal cord. The indifferent electrode was clamped to a spinous process. The action potentials were recorded from the deep fibular nerves and the gastrocnemius nerves (on both sides) with the aid of a condensor-coupled amplifier and a cathode ray oscillograph. The threshold values for the action potentials in the different nerves were determined at varying positions of the stimulating point, the needle being pushed through the spinal cord with aid of a micrometer gauge. The formol-fixed spinal cord was frozen and direct studies made of serial sections (100 μ thick). The channel made by the needle electrode was easily reconstructed from these preparations, (see Fig. 1 and 2).

Results

Stimulation of different points at the boundary between the L_7 and S_1 segments is followed by potential responses in the deep fibular and gastrocnemius nerves, the different latencies of which indicate both direct as well as mono- and multisynaptically transmitted impulses (cf. RENSHAW 1940). This paper only deals with the potential waves due to direct stimulation of different intraspinal structures of the motoneurons.

Direct volleys evoked from the anterior horn.

The lowest threshold values for direct volleys in the different nerves are generally obtained when that part of the white matter which contains ventral root fibres is stimulated. Dorsally the threshold is somewhat higher. Within the *lateral* part of the anterior horn the low threshold region for the gastrocnemius nerve lies dorsally to the corresponding area for the deep fibular nerve. There are many indications that the cell-bodies of the motoneurons to the gastrocnemius nerve lie in the posterolateral cell-column of the anterior horn.

Fig. 1 shows the diagrams from two representative experiments (A and B) which may indicate a difference in the intraspinal course of the motoneuron fibres to the different nerves. The threshold values for direct volleys in the gastrocnemius nerve (solid line) and the deep fibular nerve (broken line) are plotted against the depth of the needle point in mm. from the dorsal surface. The course of the channels (A and B) made by the needle in the two experiments is illustrated below the diagrams. The displacement to the right of the curve for the deep fibular nerve in relation to the curve for the gastrocnemius nerve thus may indicate that the cell-bodies of motoneurons belonging to antagonistic systems have a different orientation in the dorsoventral plane. Further studies of the neighbouring segments are necessary to solve this problem.

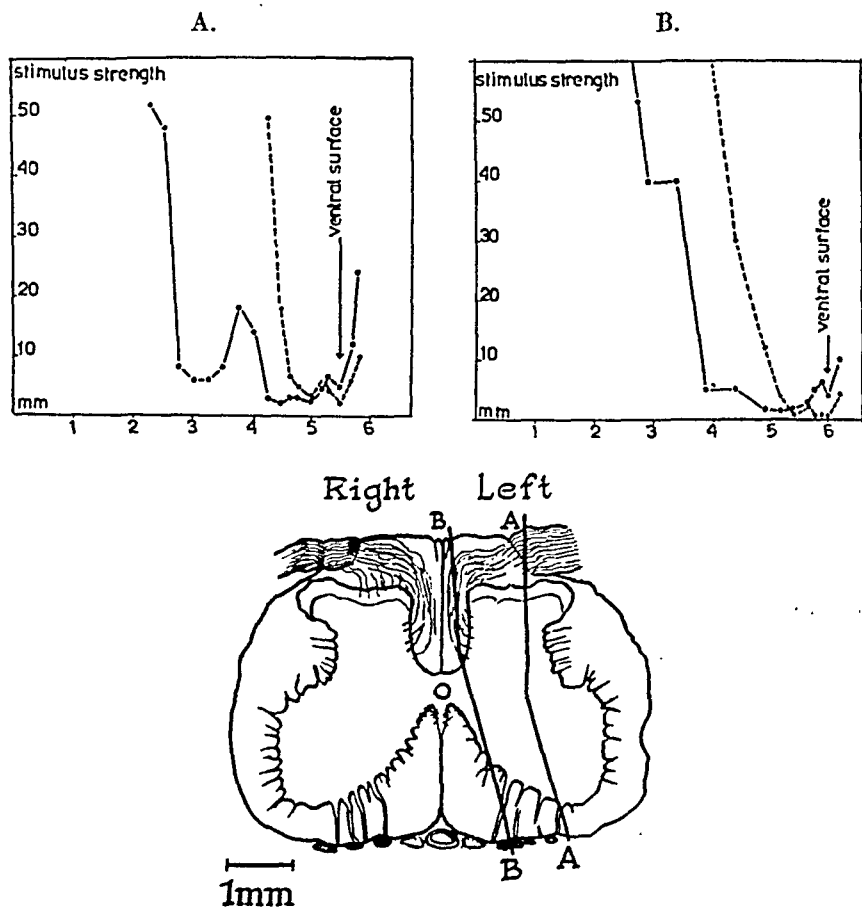


Fig. 1. A and B, threshold curves for direct volleys in the gastrocnemius nerve (solid line) and the deep fibular nerve (broken line) on the left side (two experiments). The strength of the threshold stimulus in arbitrary units (vertical axis) is plotted against the distance between the stimulating needle point and the dorsal surface of the spinal cord (in mm.; horizontal axis). The sketch below illustrates the two channels made by the needle in the two experiments (A and B). The figure is microscopically reconstructed from serial sections. Boundary between the L_7 and S_1 segments.

Direct volleys evoked from other areas than the anterior horn.

At an early stage in the investigations it was observed that direct responses could be evoked from the dorsal region of the spinal cord. Actually, it became apparent that direct volleys with relatively low thresholds could be evoked from widespread

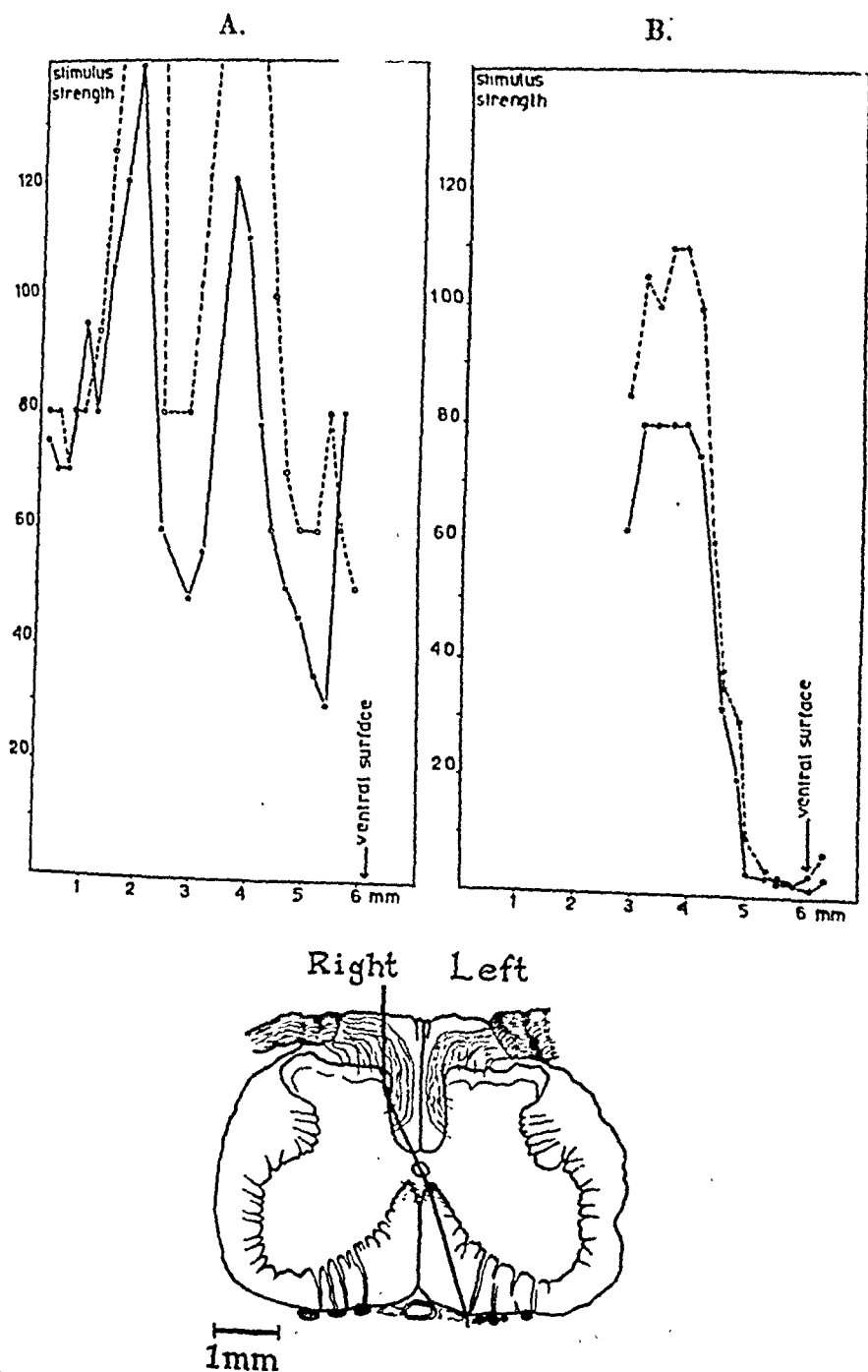


Fig. 2. Threshold curves (cf. Fig. 1) for direct volleys in the gastrocnemius nerve (solid line) and the deep fibular nerve (broken line) on the right (A) and left (B) side. The channel made by the needle, is illustrated in the figure below the diagrams. Same animal and cord section as in Fig. 1.

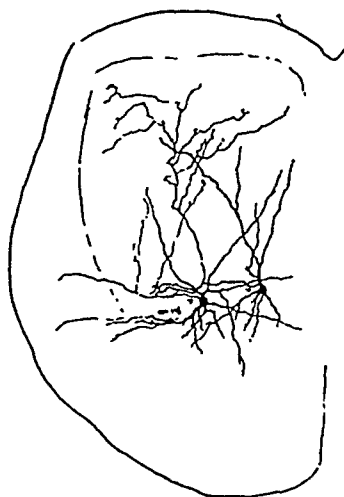


Fig. 3. Two anterior horn cells with dendrites. Mouse, spinal cord, lumbar segment, GOLGI method (from an unpublished figure provided by the kindness of Dr. RAFAEL LORENTE DE NÓ).

regions within the segment under observation. The shape of the threshold curves does, however, vary considerably depending on the path followed by the stimulating electrode on its passage through the spinal cord. This fact refutes the supposition that the motoneurons are stimulated by direct current spread to the anterior horn. Further, a difference in latency (about 0.3 msec.) has been found for the direct responses evoked from dorsal and ventral regions. Neither can the direct potential waves evoked from the dorsal region be transmitted in afferent fibres since the dorsal roots L_5 to S_2 had been cut. The observations, therefore, favour the assumption that the dendrites of the motoneurons are locally excited.

Fig. 2 shows the different shape of the threshold curves obtained from the gastrocnemius (solid line) and deep fibular nerves (broken line) on the right (A) and left (B) side, when the needle electrode passes from the right to the left and penetrates the commissures. The pictures below the diagrams in Fig. 2 show the channel of the needle electrode. It should be pointed out that the distance between the needle point and the dorsal surface should be corrected due to the depression of the spinal cord under the electrode. Experiments have shown that this error is limited

to the moment when the needle penetrates the dorsal surface of the cord.

The three dips of the curves in 2 A indicating low threshold values correspond to: 1) right dorsal root entrance zone, 2) posterior commissure and the dorsally situated part of the right posterior funiculus, 3) left anterior funiculus. The two crests correspond to; 1) the medial area of the right posterior horn penetrated by the needle and 2) the central canal.

In addition direct responses could also be evoked from the lateral funiculi, *i. e.* from an area containing presynaptic collaterals. Effects have been obtained from the nerves on one or both sides following ipsi- as well as contralateral stimulation.

Fig. 3 is a picture from the collection of Dr. LÓRENTE DE NÓ which shows the dendritic branches from two anterior horn cells. The dendrites obviously show a general tendency towards the posterior, anterior and lateral parts of the spinal cord (cf. CAJAL 1909) and in the above experiments it is interesting to note that relatively low threshold values are obtained from the posterior, anterior and lateral funiculi. In addition the above experiments also indicate dendritic branches towards the contralateral part of the spinal cord (see Fig. 2).

Discussion

The steep slopes and the varying shape of the threshold curves indicate great selectivity in the method of stimulation. A relatively strong stimulus is generally required in order to evoke nerve volleys from the dendrites. This would seem to indicate that it is necessary to create a current field of a certain extensiveness and density in order to stimulate a sufficient number of the fine dendritic branches thus producing a discharge from the motoneurons. Stimulus strength then does not only indicate the thresholds of the single dendritic fibres but may also reflect the density of the stimulated units. Consequently the curves in Fig. 2 A may reflect the relative density of the dendrites in different regions.

The above experiments show that it is possible to stimulate different parts of the motoneurons and indicate that the dendritic

branches are principally localised to areas containing presynaptic collaterals. The physiological significance of the correlation between the orientation of collaterals and motoneuron dendrites is obvious.

It is of great interest to compare these results with CAJAL's description of the motoneuron dendrites. In his classical work on the histology of the central nervous system CAJAL demonstrated the copious branching of the dendrites and described their general orientation. Starting with a purely morphological description he gave a physiological interpretation of the dendritical branching which in a surprising manner agrees with the conclusions to be drawn from the present experiments. »On ne peut, en effet, s'empêcher de penser que la diversité topographique de leurs nombreuses dendrites a pour but de mettre des segments différentes de l'appareil protoplasmique ou récepteur de la cellule au contact de chacune des espèces de collatéraux qui circulent dans la corne antérieure.» (CAJAL 1909).

Summary

1. Experiments have been performed in which microstimulation (needle point not exceeding 0.03 mm) of the spinal cord at the boundary between the L_7 and S_1 segments was used. The relative threshold values for direct potential waves recorded from the gastrocnemius and deep fibular nerves (of both sides) were measured at different depths of the stimulating needle point.

2. The experiments show that local stimulation of different intraspinal structures of the motoneurons is possible.

3. Relatively low threshold values obtained from the posterior, anterior and lateral funiculi may indicate the localisation of dendritic bundles within these regions.

4. The distribution within the anterior horn of the low threshold areas for the gastrocnemius and deep fibular nerves may indicate different localisation of the motoneurons belonging to antagonistic systems. Other interpretations are not excluded.

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